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Differential expression of photosynthetic genes amongst two different leaf ages during low temperature acclimation in *Brassica napus* cv. Jet Neuf

by

MINATI SINGH ©

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

IN

Plant Molecular Biology
DEPARTMENT OF AGRICULTURAL, FOOD AND NUTRITIONAL SCIENCE

EDMONTON, ALBERTA
Fall 1995

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Differential expression of photosynthetic genes amongst two different leaf ages during low temperature acclimation in *Brassica napus* cv. Jet Neuf** submitted by Minati Singh in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Plant Molecular Biology**.

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Date: 27th July 1995

To

my beloved husband

Madhuvrut

and

my darling daughter

Munmun

ABSTRACT

The effect of low temperature acclimation on differential gene expression between the chloroplast and nucleus in *Brasscia napus* was examined. Two different leaf ages were studied: mature leaves that underwent little cell division during acclimation and attained relatively lower levels of freezing tolerance and young leaves that were undergoing active cell division and attained relatively higher levels of freezing tolerance. Gene expression, protein accumulation and gene methylation were examined in these leaves. Nuclear and chloroplastic rRNAs, and four photosynthetic genes were used (nuclear encoded: *cab* and *ssu* and chloroplast encoded: *lsu* and *psbA*). In mature leaves, acclimation was associated with increased rRNA expression and decreased expression of both chloroplast and nuclear encoded photosynthetic genes. Methylation was examined as a possible mediator of gene expression. Hypomethylation of chloroplastic DNA could be correlated with increased rRNA transcription. While a similar relationship was noted between methylation of the photosynthetic genes and acclimation, examination of transcript abundance indicated there was no clear relationship between hypomethylation and increased transcript abundance. The low transcript levels, lack of protein turnover, low growth rate and the the drop in chlorophyll levels, suggests that chloroplasts are not differentiating in the mature leaves during acclimation. In contrast to the mature leaves, young leaves were actively growing during the acclimation time. Increases in transcript levels of ribosomal and photosynthetic genes were noted. In general, protein levels reflected the transcript levels. These results may suggest that the chloroplasts were differentiating during acclimation. Methylation was correlated with acclimation and leaf development. Examination of transcript levels showed that there was a positive relationship between *ssu* transcript levels and the degree of gene methylation. In conclusion, this study shows that different leaf ages undergo different changes in development, gene expression and methylation during low temperature acclimation. Examination of individual genes shows both positive and negative relationships between expression and methylation status.

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LIST OF ABBREVIATIONS

bp	Base pair
BSA	Bovine serum albumin
CAB	Chlorophyll a/b binding protein
cDNA	Complementary deoxyribonucleic acid
COR	Cold regulated
cpm	Counts per minute
CTAB	Cetyltrimethyl ammonium bromide
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid
Kd	Kilodalton
LSU	Large subunit of RUBISCO
MOPS	4-Morpholinepropanesulfonic acid
mRNA	Messenger ribonucleic acid
NaOAc	Sodium acetate
NTP	Nucleotide tri-phosphate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBF Percoll	Polyethylene Bovine Serum Albumin Ficoll Percoll
PMSF	Phenylmethylsufonyl fluoride
RFT	Relative freezing tolerance
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RUBISCO	Ribulose bisphosphate carboxylase-oxygenase
SDS	Sodium dodecyl sulphate
SSC	Standard sodium citrate solution
SSU	Small subunit of RUBISCO
TAE	Tris acetate EDTA
TCA	Trichloroacetic acid
TE	Tris-EDTA buffer
Tris	Tris (Hydroxymethyl) methylamine

A	Adenine
T	Thymine
G	Guanine
C	Cytosine

CHAPTER 1

INTRODUCTION

Extreme environmental conditions severely limit the geographical distribution of plants. These extreme conditions account largely for the discrepancies between the potential and the actual crop yields. Many plants, however, have evolved mechanisms that allow them to acclimate to various environmental stresses (Key and Kosuge, 1984; Levitt, 1972). Freezing is one such environmental stress. One of the most dramatic manifestations of cold acclimation, or cold hardening is increased freezing tolerance. In this condition the cells resist external freezing without injury (Levitt,1980; Sakai and Larcher, 1987). Non-acclimated wheat, for example, is killed at temperatures near -5^0C, whereas cold acclimated wheat survives at temperatures down to -20^0C (Zvereva and Turnova, 1985).

A number of changes have been associated with cold acclimation. Common examples include the appearance of new isozymes, increased sugar and soluble protein content, and increased levels of proline and other organic acids (Levitt, 1980; Sakai and Larcher, 1987; Steponkus, 1984). In addition, membranes are involved in increased freezing tolerance of acclimated plants (Steponkus, 1984; Steponkus and Lynch, 1989 and Palta *et al*, 1993). The precise role each change has on cold acclimation is not known.

Cold acclimation involves changes in gene expression in plants (Weiser, 1970). Both increased and decreased levels of mRNAs and specific polypeptides have been observed during cold acclimation (Glimour *et al*, 1988; Guy *et al*, 1985; Guy and Haskell, 1987; Meza-Basso *et al*, 1986; Mohapatra *et al*, 1987; Robertson *et al*, 1987). At this time, the functions of the proteins preferentially synthesized at low temperature are unknown.

The induction and maintenance of a cold acclimated state is an energy requiring process. During cold acclimation, a net accumulation of many metabolites has been documented (Levitt, 1980). The metabolic energy and carbon skeleton necessary to support this accumulation of metabolites comes primarily from photosynthesis (Gusta and Fowler, 1979; Kacperska-Palacz,

1978). Thus, the ability to adapt to low temperature is, in part, related to the ability to maintain relatively high levels of photosynthesis at low temperatures (Steffan and Palta, 1986).

Other workers have suggested that differences in freezing tolerance can be correlated with developmental stage or leaf age. This study sets out first to determine whether such a difference exists and then determines how two very different leaves, the mature, expanded leaf and the young, expanding leaf, control and co-ordinate gene expression between the nucleus and the chloroplast during low temperature acclimation.

Brassica napus cv. Jet Neuf was chosen as the plant model for this investigation. As a winter cultivar it serves as an excellent system for studying the changes in gene expression during low temperature acclimation. The first fully expanded mature leaves and the fourth young expanding leaves were used to examine the co-ordination of gene expression between the chloroplast and the nucleus during low temperature acclimation. Both chloroplastic rDNA (pUC11-5) and nuclear rDNA (pMF2) probes were used to study the changes in the expression of ribosomal machinery during low temperature acclimation. Four photosynthetic genes were used. Two of the genes encode products involved in photosystem II (PSII). Of these *cab* is nuclear encoded (chlorophyll a/b binding protein) and the other gene, *psbA* (32 Kd Quinone binding protein) is chloroplast encoded. Their gene products are membrane bound proteins of the thylakoid. The other two genes used in this study are the chloroplast encoded *lsu* (large subunit of RUBISCO) and nuclear encoded *ssu*, (small subunit of RUBISCO). The two subunits of RUBISCO are located and remain assembled in the stroma of the chloroplast.

Three hypotheses were proposed:

1. Leaf development does not affect freezing tolerance.

2. Photosynthetic gene expression is co-ordinated during acclimation.

3. Methylation of genes is not associated with leaf development or acclimation.

In order to test these three hypotheses four objectives were laid out. These objectives of my research were:

1. To assess the freezing tolerance of mature and young leaves of *Brassica napus* and to find an appropriate time period when there was the greatest difference in freezing tolerance between acclimated and non-accclimated leaves and freezing tolerance was maximal in both leaf ages. The relationship between acclimation, developmental changes and cell division were studied in mature and young leaves in order to characterize the leaves with regard to developmental stage during cold acclimation.

2. To study the rRNA expression of both nuclear and chloroplastic ribosomal genes and the possible role of methylation in relation to rRNA expression during low temperature acclimation.

3. To study gene expression of chloroplast encoded genes (*lsu* and *psbA*) and nuclear encoded genes (*ssu* and *cab*) in mature and young leaves during low temperature acclimation.

4. To study the methylation patterns of chloroplast encoded genes (*lsu* and *psbA*) and nuclear encoded genes (*ssu* and *cab*) in mature and young leaves during low temperature acclimation.

Review Of Literature

A. Differences between low temperature stress and low temperature acclimation

Many plants native to tropical or subtropical climates show more or less pronounced dysfunction in their physiological processes at chilling temperatures between 0^0C and 10^0C, and they eventually die if kept at these temperatures for an extended period. The transient physiological and biochemical adjustments induced by abrupt change in the environment leading to senescence is a stress response. Plants that experience low temperature stress may exhibit a loss of vigour and reduced growth rates in the absence of other visual symptoms of injury. These persist even after the plant returns to warm conditions. More severe low temperature stress promotes cellular autolysis and senescence (Saltveit and Morris, 1990). The loss of chlorophyll, apparent as leaf yellowing, may occur in the light as a consequence of photo-oxidation. The loss of cellular integrity gives the tissue a water soaked appearance because of the leakage of cellular fluids into the intracellular space (apoplastic spaces). Other symptoms, such as plasmolysis and loss of turgor, also imply changes in cellular membranes that ultimately result in failure to maintain cellular comparmentation. Alterations in metabolism usually precede these visual and cellular symptoms. For example, the rates of CO_2 and C_2H_2 production usually increase prior to appearance of visual symptoms. Gene expressions and protein synthesis are suppressed and do not recover even when plants are returned to warm temperatures. The damage is irreversible.

A response induced by low temperature causing a phenotypic alteration over a single generation time without any compositional change in the genetic complement is known as low temperature acclimation. The developmental response to the new low temperature environment often results in increased freezing tolerance. Plants that are able to acclimate initially respond to low temperature in much the same manner as plants that cannot acclimate. However, the stress response is less dramatic and the duration is limited. Once the plant adjusts to the low temperature i.e.

achieves a new homeostasis, it begins to attain freezing tolerance. This increased freezing tolerance is correlated with increased rRNA expression and increased protein synthesis. Other changes in metabolism have been reported. The following are examples that are discussed in detail in the next section: increases in osmotic concentrations, increases in lipid accumulation and increases in unsaturated ratty acids, increases in soluble protein content, an inverse relationship between the rate of respiration and freezing tolerance and increased photosynthetic capacity. Besides these some novel cold regulated proteins have been observed which correlates with increased freezing tolerance (Guy, 1990; Mohapatra *et al*, 1987a,b; Weretilnyk *et al*, 1993;Thomashow *et al*, 1990; Gilmour *et al*, 1990).

B. Freezing stress and freezing tolerance

When temperatures fall below the freezing point of water, ice forms in the plant tissue. This occurs first at locations having the least negative osmotic potential. Ice normally forms first in xylem vessels in leaves and stems and intercellular spaces (Levitt, 1980). Once ice forms it spreads throughout the vessel and into intercellular spaces of other tissue, but does not penetrate the intact plasma membrane to inoculate the cytoplasm. Therefore, the ice crystals enlarges at the expense of water vapor and the surface film of liquid water on the cell wall. Under these conditions, water moves out of the cell and therefore the cytoplasm dehydrates and the cells collapse. On the otherhand acclimated cells can survive extracellular ice formation. This is a result of numerous cellular changes, including increased osmotic potential and alteration of the plasmalemma.

In both acclimated and non-acclimated cells the plasma membrane assumes a critical function in the freezing process. It serves a physical barrier preventing ice nucleation of the cytoplasm from extracellular sites. Intracellular ice formation is lethal to the cell because intracellular membranes and other structures are sheared. Cells that are not acclimated cannot survive this dehydration (Levitt, 1980).

C. Low temperature induction of freezing tolerance

Developmental changes

Freezing tolerance is the ability of a plant to survive extracellular ice formation. Plant growth and development at low temperature plays an important role in controlling freezing tolerance. The potential of leaves to adjust to an altered environmental temperature appears to be governed by developmental stage, with fully mature leaves being less capable of acclimating to variation in temperature than young developing leaves (Krol *et al*, 1984; Krol and Huner, 1985; Rutten and Sanatarius, 1992; Hurry and Huner, 1991; Boese and Huner, 1990).

Fowler and Gusta (1984) established that growth of seedlings of winter-hardy cereals at low temperature was mandatory for the initiation of the cold-acclimation process and for the subsequent establishment and survival of cold hardy crown tissue. Similarly, Krol *et al* (1984) reported that maximum vegetative growth coincided with maximum freezing tolerance in cold hardened rye plants. This was not observed in the leaves of non-hardened plants. Rye plants grown at 5^0C were smaller in size as compared to the ones that were grown at 20^0C. However, there was no change in the number of leaves per plant. Growth at 5^0C also resulted in characteristic changes in leaves. Such changes include increased cytoplasmic material per mesophyll cell, epidermal and mesotome sheath cell-wall thickenings, increased leaf thickness, decreased stomatal frequency and altered distribution patterns, higher dry weight and increased chlorophyll and carotenoid per leaf area (Krol *et al*, 1984). Starch and osmophilic granules have been shown to be lost from chloroplasts during acclimation in *Solanum acuale* (Li *et al*, 1979). In addition, characteristic crimping of chloroplast thylakoids and reduced intrathylakoid space has been noted in barley grown at 2^0C or 5^0C. However, the grana size in chloroplasts of cold acclimated rye is similar as compared to non-acclimated rye (Huner *et al*, 1984). Thus, low temperature slows the rate of growth and results in characteristic morphological, anatomical and biochemical alterations as a result of the constraints imposed upon leaf development at low temperature (Huner *et al*, 1984).

In order for a plant to acclimate at low temperature, the plant must balance light energy absorbed with the metabolic capacity to utilize this energy (Osmond, 1981). Plants grown at low temperatures usually have a higher photosynthetic capacity (Steffan and Palta, 1986; Alberdi and Corcuera, 1991; Holaday *et al*, 1992). It has been suggested that low temperature induces lowering of the activation energy required for the enzymatic reactions (Oquist, 1983). Increased light and CO_2 saturated rates of photosynthesis have been reported in acclimated rye leaves (Huner, 1985; Huner *et al*, 1984).

The environmental conditions that induce freezing tolerance are potentially photoinhibitory (Huner *et al*, 1993). Somersalo and Krause (1989, 1990) showed that cold acclimated spinach plants could increase tolerance to photoinhibition. This response was dependent upon leaf ages and developmental history of the leaves. Boese and Huner (1990, 1992) showed that leaves that had expanded at low temperature acquired greater resistance to low temperature photoinhibition, whereas, the oldest, fully expanded leaves exhibited the greatest susceptibiiity to photoinhibition. Steffan and Palta (1987) showed that *Solanum* plants respond to decreased growth temperature and constant light intensity, by increasing their light energy utilization capacity and decreasing the efficiency of light energy trapping by reducing the antennae size. By balancing the light energy flux through the chloroplasts, light-dependent injury to the photosynthetic apparatus is avoided and the levels of net photosynthesis required for cold acclimation are maintained (Steffan and Palta, 1987).

Results also suggest that the photosynthetic response to low temperature is species and cultivar dependent (Huner *et al*, 1993). Exposure of fully expanded leaves of winter cereals to short-term, low temperature shifts inhibits the electron transport capacity and carbon assimilation (Huner *et al*, 1986). In contrast, growth of rye leaves at low temperature stimulates electron transport capacity and carbon assimilation (Huner *et al*, 1986). Cold tolerant evergreens show a different photosynthetic response. For example, exposure of *Pinus sylvestris* to 5^0C and short days under controlled environmental conditions causes a significant depression in photosynthesis and electron transport (Oquist *et a*l, 1980). Similar observations have been made in *Pinus*

sylvestris(Ottander and Oquist, 1991), *Hedera helix* (Oberhunder and Bauer, 1991) and *Ilex aquifolium* (Groom *et al*, 1991).

Photosynthesis and cold acclimation

During low temperature acclimation, a net accumulation of many metabolites has been well documented (Levitt, 1980). The metabolic energy and carbon skeletons necessary to support this accumulation comes primarily from photosynthesis (Gusta and Fowler, 1979). The ability to maintain relatively high levels of photosynthesis at low temperatures reflects the ability to acclimate at low temperatures (Steffen and Palta, 1986). Resistance to short-term photoinhibition (hours) in winter cereals is a reflection of the increased capacity to keep Q_A oxidized under high light conditions and low temperature. This is because of an increased capacity for photosynthesis. These characteristics reflect photosynthetic acclimation to low growth temperature and can be used to predict the freezing tolerance of cereals (Huner *et al*, 1993). It is proposed that the enhanced photosynthetic capacity reflects an increased flux of fixed carbon through to sucrose in source tissue as a consequence of the combined effects of storage of carbohydrate such as fructans in the vacuole of leaf mesophyll cells and enhanced export to the crown. Long-term exposure (months) of cereals to low temperature photoinhibition indicates that this reduction of photochemical effciency of PS II represents a stable, long-term down regulations of PS II to match the energy requirements for CO_2 fixation. Conifers (Oquist and Strand, 1986), several cereals (Oquist and Huner, 1989) and *Spinacea oleracea* (Sommersalo and Krause, 1989a, b) may undergo partial photoinhibition during prolonged cold acclimation.

Recently, Holaday *et al* (1992) reported that *Spinach oleracea* exposed to 10^0C for 10days exhibited higher rates of photosynthesis than spinach plants maintained at 24^0C. On the other hand, inhibition of photosynthesis was observed for spinach plants grown at 5^0C and subsequently shifted to 16^0C. This was observed regardless of the measuring temperature and occurred with no changes in internal CO_2 concentrations (Boese and Huner, 1990). They concluded that fully expanded spinach leaves can not acclimate photosynthetically but in fact are stressed when exposed to an abrupt change in temperature. Similar trends have been reported by Somersalo and Krause

(1989) for spinach and Maciejewska *et al* (1984, 1987) for winter rape subjected to low temperature shifts. Employing additional controls, Boese and Huner (1990) showed that aging during the shift period can account for most of the observed reduction in the rate of photosynthesis. The capacity to acclimate photosynthetically to low temperature appears to be dependent upon the physiological age of the tissue and the developmental history of the leaf (Krol and Huner, 1985).

Decreasing temperature leads to decreases in the irradiance required to saturate photosynthesis (Berry and Bjorkman, 1980; Falk et al, 1992). This has been interpreted to reflect the accumulation of photosynthetic metabolites at low temperatures and light saturating irradiance (Stitt and Grosse, 1988). Evidence indicates that low temperature induced O_2 insensitivity of CO_2 fixation reflects inorganic phosphate limitation of photosynthesis (Leegwood, 1985; Stitt et al, 1987). This may, in part, result from the fact that optimal rates of photosynthesis at low measuring temperature require higher inorganic phosphates concentrations than photosynthesis at moderate to high temperatures (Leegwood, 1985). Exposure to low temperatures results in accumulation of phosphorylated metabolites such as hexose phosphates thus reducing the available inorganic phosphates in the stroma for photosynthesis at low temperatures (Labate *et al*, 1990). The rise in phosphoglycerate/triose phosphate (PGA/TP) favours starch accumulation within the chloroplast of spinach and wheat over sucrose synthesis in the cytoplasm (Stitt *et al*, 1990). This could be the result of restriction on photosynthetic electron transport and thus, decreased capacity to generate ATP and NADPH. As a result CO_2 fixation is limited. In contrast to spinach and wheat, the ratio of PGA/TP remains constant with low temperature. Furthermore, it has been suggested that maximal rates of photosynthesis occurs at the level of the inorganic phosphate translocator rather than the sucrose synthesis. The accumulation of excess photosynthetically fixed carbon in the form of sucrose during cold acclimation and its positive correlation with cold tolerance have been reported (Levitt, 1980; Guy *et al*, 1992). If exposure to low temperature predisposes plants to inorganic phosphate limitation of photosynthesis (Sharkey *et al*, 1986; Stitt *et al*, 1987), cold tolerant plants that grow at low temperature and exhibit increased rates of photosynthesis must overcome this limitation in some way.

Biochemical changes

Levels of RNA, proteins, amino acids, membrane lipids, and soluble sugars are altered during freezing tolerance (Siminovitch *et al*, 1968). Along with these changes, the plasmamembrane undergoes compositional (Umeura and Yoshida, 1984; Lynch and Steponkus, 1984) and qualitative changes during cold hardening (Niki and Sakai, 1981; Fennell and Li, 1986). The biochemical changes observed during freezing tolerance suggest that changes in nucleic acids and proteins may also play a role in freezing tolerance.

The correlation between DNA levels and induced freezing tolerance is inconsistent. Increases in DNA content have been reported in winter wheat (Teraoka, 1973), apple twigs (Li and Weiser, 1969) and suspension cells of *Brassica napus* cv. Jet Neuf (Johnson-Flanagan and Singh, 1987, 1988) during the induction of freezing tolerance. A decrease in DNA content has been shown in potato during low temperature acclimation (Chen and Li, 1980), while constant DNA levels have also been observed. For example, boxwood leaves (Brown and Sakai, 1972), black locust (Siminovitch, 1963), red osier dogwood (Li and Weiser, 1967) and mimosa epicotyl and hypocotyl (Brown and Sakai, 1972) all maintain constant DNA levels.

Generally, total RNA increases during the induction of freezing tolerance (Sarhan and Chevrier, 1984; Siminovitch *et al*, 1967; Oslund and Li, 1972; Zvereva and Turnova, 1985; Sarhan and D'Aoust, 1975). Positive correlations between increased RNA content and increased freezing tolerance have been reported in potato (Oslund and Li, 1972) and wheat (Sarhan and Chevrier, 1984). This increase in RNA content reflects changes in the population of rRNA and soluble RNAs (mRNA and tRNA).

Increased RNA levels were thought to be responsible for increases in freezing tolerance (Brown and Sasakai, 1972). Certainly, numerous workers reported such increases (Brown and Sasakai, 1972; Chen and Li, 1980; Sarhan and Chevrier, 1985). Increases in RNA polymerase I activity (Sarhan and Chevrier, 1985) and rDNA cistron number (Paldey and Devay, 1983a) have been shown to control rRNA levels. Sarahan and Chevrier, (1985) observed a 3 fold increase in RNA polymerase I activity in the hardened winter wheat as compared to a spring cultivar that had been exposed to acclimating conditions supporting the fact that rRNA synthesis was induced during low temperature

acclimation. Paldey and Devay (1983a) noted increases in rDNA cistrons with acclimation leading to increases in rRNA content. There were different classes of RNA present, and one that was transcribed during low temperature acclimation. Interestingly, the same class was decomposed when shifted to higher temperature. This lead to the assumption that different cistrons may be transcribed during low temperature acclimation (Paldey and Devay, 1983b). Furthermore, application of a transcription inhibitor, actinomycin D, resulted in 80% reduction of RNA synthesis (Paldey and Devay, 1977) with a concomitant decrease in freezing tolerance. This suggested that *de novo* synthesis of RNA was involved.

Increases in ribosome content have been reported during low temperature acclimation in black locust seedlings (Siminovitch *et al*, 1963) and potato (Chen and Li, 1980). In addition, polysome size increases with acclimation (Chen and Li, 1980). This may reflect greater stability (Brown and Brixby, 1972). Laroche and Hopkins (1987) suggested that differences in ribosomal polypeptides could alter the stability of ribosomal structures (Laroche and Hopkins, 1987). Such differences have been shown in three acidic ribosomal polypeptides from cold-hardened and non-hardened winter rye. Slight change in isoelectric point and relative molecular weights led to altered stability of the ribosome structure. These changes may allow protein synthesis to proceed at low temperatures (Laroche and Hopkins, 1987). Brown and Bixby (1972) reported an increase in monomer stability of the ribosomes with acclimation. They also suggested that ribosomal changes could alter protein synthesis by allowing the translational selection of messages.

Generally the soluble proteins increase during the induction of freezing tolerance. This increase has been shown in black locust (Brown and Brixby, 1975), red pine (Pomeroy *et al* , 1970), boxwood (Gusta and Weiser, 1972), alfalfa (Gerloff *et al* ,1967), cereals (Pomeroy *et al*, 1970; Chou and Levitt, 1972; Sarhan and Chevrier, 1985) and potato (Chen and Li, 1980). Qualitative changes in soluble proteins, such as structural changes and isozyme changes, have been reported to be associated with induction of freezing tolerance. It has been proposed that exposed SH groups form S-S bonds between protein molecules during freeze induced dehydration. In

acclimated plants, SH groups are not exposed in the proteins and, therefore, the enzymes have greater stability than that of non-acclimated (Levitt, 1972).

Cold hardening of cabbage has been shown to induce changes in RUBISCO with the formation of two distinct forms of each of the subunits (Shomer-Ilan and Waisel, 1975). The structural and kinetic properties of this enzyme have been shown to change when rye is cold hardened leading to increased stability to low temperature (Huner and MacDowall, 1978, 1979), brought about by an *in vivo* conformational change in the SH reactive groups (Huner *et al*, 1981). Comparison between acclimated *Solanum commersonii* and non-acclimated *Solanum tuberosum* also show that RUBISCO (and possibly other soluble enzymes) has the ability to acclimate to low temperature (Huner *et al*, 1981). Further, alteration of the amino acid content of cytoplasmic proteins, leading to changes in water retaining properties has been shown (Rochart and Therrien, 1975). These results indicate that protein/enzyme changes may play some role in controlling adaptation to temperature changes (reviewed by Graham and Patterson, 1982).

Inhibition of protein synthesis by cycloheximide has been shown to prevent both the induction of freezing tolerance and the formation of rough endoplasmic reticulum (Hatano *et al* , 1976; Niki and Sasakai, 1983). Therefore, this indicates that the freezing tolerance is intimately associated with RNA and protein synthesis. Synthesis of cold regulated polypeptides (COR) closely parallels acclimation. That is, the appearance of COR polypeptides generally coincides with the onset of freezing tolerance and their synthesis continues as long as the plants are kept in cold acclimating temperatures, but declines quickly under deacclimating conditions. The results of Guy and Haskell (1987) have clearly demonstrated these points. Their *in vivo* radiolabelling studies revealed that 160-, 117-, 85-Kd COR polypeptides were synthesized within 24 hr of exposure to cold temperature and that they continued to be synthesized for as long as the plants were kept in the cold. Further, it was found that returning the plants to 25^0C resulted in rapid cessation of synthesis of these polypeptides. A similar relationship between COR polypeptide synthesis and acclimation and deacclimation have been observed in alfalfa (Mohapatra *et al*, 1987a,b) and *Arabidopsis* (Kurekella *et al* , 1988).

The alterations in polypeptide synthesis that occurs during cold acclimation involve changes in the mRNA populations. The first evidence came from the work of Guy *et al* (1985). In their studies they used *in vitro* translation assays to show that poly (A)+ RNA from 2 day acclimated plants produced polypeptides of 180 and 82 kDa that were not synthesized by poly (A)+ RNA isolated from nonacclimated plants. Subsequently, cold-acclimation-associated changes in mRNA populations has been examined in a number of other plant species using *in vitro* translation assays, and the results were similar to those obtained in *Spinach oleracea*: Gilmour *et al* (1988) and Thomashow *et al* (1990) obtained evidence of increases in translatable mRNAs encoding polypeptides of 160, 47, 24, and 15 kDa in cold acclimated Arabidopsis; Kurkela *et al* (1988) have also shown increases in translatable mRNAs for polypeptides of 150, 45, and 24 kDa in 24 hr cold acclimated Arabidopsis; Mohapatra *et al* (1987a,b) found increases in translatable mRNAs in cold-acclimated *Medicago falcata* and *Medicago sativa.*; Cattivelli and Bartells (1993) isolated cold regulated transcripts from barley; and Weretilnyk *et al*, (1993) identified three low temperature-regulated transcripts from winter *Brassica napus* cv. Jet Neuf.

Whether *cor* gene expression correlates with the degree of freezing tolerance attained was first addressed by Mohapatra *et al* (1989). In studies of cold acclimated alfalfa they found that the correlation between the level of expression of pSM2201 *cor* gene and the degree of freezing tolerance attained by different cultivars was high. Similar high positive correlation coefficients were obtained for other two *cor* genes pSM784 and pSM2358. The result of Mohapatra *et al* (1989) indicate that a link exists between the expression of alfalfa *cor* genes and the level of freezing tolerance obtained by the alfalfa cultivars. However, the nature of the observed link between *cor* genes and freezing tolerance remains to be established.

Physical properties of lipids, structural proteins and enzymes, have been shown to change with cold acclimation. In recent years, ultrastructural and biophysical alterations of the plasma membrane during lethal extracellular freezing have been elucidated. Irreversible conversion of the planar membrane bilayer to tightly appressed multilamellar vesicles has been observed to be associated with lethal extracellular freezing (Gordon-Kamm and Steponkus, 1984b; Pearce and Willison, 1985; Singh *et al*, 1987). In the

case of isolated protoplasts, a hexagonal II phase has been observed, in addition to multilamellar vesicles (Gordon-Kamm and Steponkus, 1984b).

Following cold acclimation, only small changes in fatty acid unsaturation and sterol content has been observed; the phospho-lipid-to-protein ratio increases and some differences in the polypeptide composition have also been observed. The fluidity of the membranes during cold acclimation has been reported to change, with increases in phospholipids and unsaturation of fatty acids (Clarkson *et al*, 1980; Quinn and Williams, 1978; Shomer-Ilan and Waisel, 1975; Smolenska and Kuiper, 1977). Some studies report increases in the proportion of unsaturated fatty acids (de la Roche *et al* 1972; Grervier *et al* 1972; Willmont 1975, 1977). However, others have failed to detect such an increase in fatty acid unsaturation during cold acclimation (de la Roche *et al* 1975; de la Roche *et al* 1979; Vigh *et al* 1985). The contradictory results can be explained in part by the fact that many researchers have analyzed the lipid composition of whole tissue or crude membrane, rather than purified plasma membrane.

In addition, there is more membrane material present and available for plasmalemma expansion during de-plasmolysis (Dowgert and Steponkus, 1984) and cells (Johnson -Flanagan and Singh, 1986). The nature of this reservoir of membrane material is not known. However, increases in smooth and rough endoplasmic reticulum membrane and vesicles have been shown during cold acclimation (Niki and Sakai, 1983; Orr *et al*, 1986; Pomeroy and Siminovitch, 1971; Singh *et al*, 1987; Srivastava and O'Brien, 1966). These vesicles have been observed to fuse with plasmalemma and have been proposed as a mechanism of plasmalemma compositional alteration during cold hardening.

D. Regulation of chloroplast gene expression

Chloroplast DNA ranges from 85kb to more than 190kb depending on the organism from which it is derived, but for most plants examined it is around 150kb (Bohnert *et al*, 1982). To date, there is no evidence that mRNAs can enter chloroplasts from the cytosol, so it is assumed that chloroplast mRNAs are all transcribed from chloroplast DNA. Control of transcription in chloroplast genes is prokayotic in nature (Bedbrook *et al*, 1979; Bowman *et al*,

1981). Initiation of protein synthesis occurs with formyl-methionine, as in bacteria, whereas methionine carries out this function in the cytosol. Shine-Dalgarno sequences are present just before the translational start site (Erion *et al*, 1981; Bedbrook *et al*, 1979).

Development of a functional leaf chloroplast is generally accompanied by transitory or stable increases in mRNAs pools for protein subunits of the thylakoid membrane complexes (Gruissem, 1989a; Gruissem, 1989b; Gruissem *et al*, 1989; Gruissem and Schuster, 1985). Analysis of RNA levels for different chloroplast genes in roots, hypocotyl, and cotyledons of dark-grown seedlings (Deng and Gruissem, 1988), during seed germination (Degenhardt *et al*, 1991; Dietrich *et al*, 1987) and in monocot leaves developing in the dark (Baumgartner *et al*, 1989; Mullet and Klein, 1987) have shown that temporal and organ specific plastid RNA levels are clearly a function of the plants developmental program. At the appropriate developmental stage or under certain experimental conditions, light can induce leaf chloroplast development and cause changes in chloroplast RNA levels (Deng and Gruissem, 1987; Gamble *et al*, 1988; Mullet and Klein, 1987; Schubar *et al*, 1990). The extent of transcriptional and post transcriptional regulation of plastid RNA levels during chloroplast biogenesis most likely depend on many factors, and may vary between different plant species (Bejarno and Lichtenstein, 1992; Chory *et al*, 1989; Chory and Peto, 1990). The results presently available are very limited, and, therefore, it is difficult to draw any general conclusion. However, chloroplast gene expression is regulated, in part, at the transcriptional level. Developmentally controlled changes in mRNA stability, translational activity and protein phosphorylation also play an important role (Mullet, 1988; Gruiessem, 1989). Finally, the nuclear genome is critical for the co-ordination and control of chloroplast functions (Ellis, 1981). The temporal, spatial and environmental control of chloroplast functions, therefore, requires a detailed understanding of the mechanism by which chloroplast gene expression is regulated (Ellis, 1981; Gruissem and Tonkyn, 1993).

The abundance of chloroplast RNAs can vary substantially for different genes as a function of developmental process and environmental signals such as light (Gruissem *et al*, 1989a; Gruissem *et al*, 1989b; Mullet, 1988). Differences in RNA accumulation are controlled in part at the transcriptional

level, but the mechanism that governs the differential transcription of chloroplast genes is currently not well understood. The term differential transcription has been used to describe both the transcriptional activity of chloroplast genes controlled by their promoter strength, as well as modulation of transcription initiation frequency at individual promoters and the changes in plastid RNA polymerase activity during leaf development or after illumination. These processes may be interdependent to some extent, but for the most part they act independently of each other. It has been suggested that changes in chloroplast DNA copy number are not critical for this control (Mullet and Klein, 1987).

Transcriptional regulation of chloroplast genes

Promoter mutagenesis, in combination with comparative analysis of transcription initiation efficiency has shown that the promoter strengths in the chloroplast genome differ (Gruissem and Zurawski, 1985). *In vitro* transcription studies have shown that transcription initiation at chloroplast promoter regions respond to changes in superhelical density of the DNA (Russel and Bogorad, 1987; Stirdivent *et al*, 1985). This could be controlled by the concerted action of topoisomerase I and gyrase present in chloroplast extracts (Lam and Chua, 1987). The relevance of DNA topology for the transcription of plastid genes remains unclear, but must be considered as a regulatory factor because of multimeric, circular and superhelical structure of plastid DNA (Deng *et al*, 1989; Kolodner *et al*, 1976; Kolodner *et al*, 1975). Promoter activity could also be controlled by regulatory DNA-binding proteins that bind to the catalytically active core of RNA polymerase, thereby determining the specificity of transcription initiation. However, results favour promoter strength as the principal regulatory factor.

Regulation of transcription by DNA methylation is a controversial issue (Cedar, 1988; Dyann, 1989). It has been suggested as a mechanism for transcriptional regulation of several nuclear genes in plants (Kunze *et al*, 1988; Langdale *et al*, 1991). Recently, the control of transcription by DNA methylation has been suggested for several chloroplast genes. Methylated DNA has generally not been detected in chloroplast DNA with the exception of *Chlamydomonas*, where methylation has been shown to play a role in maternal inheritance of chloroplast DNA (Feng and Chiang, 1984; Sagar *et al*,

1981). However, Kobayaschi *et al*, (1987), Macherel *et al*, (1986) Ngernprasirtisiri,(1990), Ngernprasirtisiri *et al*, (1989), Ngernprasirtisiri *et al*, (1988a), Ngernprasirtisiri *et al*, (1988b) have reported methylation of cytosine (5mC) and very low levels of adenosine methylation (6mA) in plastid DNA. This has been correlated with transcriptional inactivation of plastid genes in *Sycamore* amyloplasts, tomato fruit chromoplasts and maize bundle sheath and mesophyll chloroplasts. *In vitro* transcription assays have shown that hypomethylation is correlated with active transcription of the 16S RNA and *psbA* genes (Kobayashi *et al*, 1990). A strict inverse correlation between the relative abundance of the transcripts of *ppc* and methylation at *Eco RII* sites in the regions containg the *ppc* genes has been reported (Ngernprasirtsiri *et al*, 1989). It is not known whether methylation led to transcriptional inactivation of genes, or occurred as a consequence of transcriptional inactivation. It is also possible that site specific methylation of other sequences recognized by different enzymes could be involved in methylation correlating with transcript abundance. Thus, it is presently unclear if DNA methylation has any role in the transcriptional inactivation of chloroplast genes.

Other studies have not shown a relationship between methylation and transcription. For example, studies on methylation pattern differences between chloroplast DNA in developing maize endosperm and mesophyll chloroplast DNA showed that the two chloroplast DNAs were indistinguishable from each other (McCullough *et al*, 1992) and this lack of distinguishable differences at the restriction site was consistent with other studies in nongreen plastids in pepper and tomato fruit, daffodil flowers and wheat endosperm (Cately *et al*, 1987; Gounaris *et al*, 1986; Hunt *et al*, 1986; Piechull *et al*, 1985; Ngernprasirtsiri *et al*, 1988).

Post-transcriptional control of plastid mRNA accumulation

Regulation of mRNA processing and stability has been widely recognized as an important control mechanism for the expression of many prokaryotic and eukaryotic genes. Genetic and molecular dissection of several mRNAs whose stability is differentially regulated has revealed RNA sequence and structural motifs that are critical for the stability and/or

turnover of the transcripts (Brawerman, 1989; Ross, 1989; Shyu *et al*, 1991; Theil, 1990; Wisdom and Lee, 1991).

Direct measurements of changes in chloroplast mRNA turnover rates are technically difficult in vascular plants, as most chloroplast mRNAs are initially transcribed into polycistronic transcripts (Barkan, 1988; Hudson *et al*, 1987; Kirsch *et al*, 1986; Rock *et al*, 1987; Sugiura, 1991; Westhoff *et al*, 1985; Westhoff and Hermann, 1989), therefore, the half-life of individual RNAs is also a function of precursor RNA half-life and the rate of processing. Further, results from polysome association studies show that *lsu* and *psbA* mRNA are less stable when bound to polysomes as compared to polysome bound depleted mRNAs. This suggests that ribosome binding and/or translation of *psbA* and *lsu* mRNAs may modulate the rate of mRNA decay in chloroplasts.

Klaff and Gruissem, (1991) showed that mRNA stability plays an important role in chloroplast mRNA accumulation. Application of transcription inhibitors such as actinomycin D to intact mature and young leaves of *Spinach olerecea* followed by quantitation of plastid RNA levels showed that the half life of *psbA* and *lsu* are similar in young leaves and that the relative transcription activities of *psbA* and *lsu* genes are similar in young and mature leaves (Klaff and Gruissem, 1991; Deng and Gruissem, 1987).

Most polycistronic transcripts in chloroplasts undergo extensive RNA processing to yield tRNAs, ribosomal RNAs and monocistronic mRNAs. Fully processed mono-or dicistronic RNAs generally represent the predominant pool of chloroplast RNAs or polysomal mRNAs, but polycistronic mRNAs can be assembled into polysomes before processing is complete (Barkan, 1988).

Several mRNAs for photosynthetic proteins have multiple 5' ends, which at least for some mRNAs, results from specific cleavage of the primary transcript (Crossland *et al*, 1984; Gatenby *et al*, 1981; Haley and Bogorad, 1990; Mullet *et al*, 1985; Sexton *et al*, 1990; Deng and Gruissem, 1988; Hanley-Baudoin *et al*, 1985; Stern and Gruissem, 1987; Westhoff *et al*, 1985). Although the significance of 5' end processing is unknown, it may provide a mechanism for translational control or differential stabilization of the mRNA (Crossland *et al*, 1984; Poulsen, 1984).

Most chloroplast mRNAs contain a unique inverted repeat (IR) sequence in their 3' untranslated region that can fold into stable stem/loop structure (Stern *et al*, 1989). These sequences are highly conserved. 3' IR sequences can cause effective termination of transcription *in vivo*. They are also *cis* acting elements for precise processing and stabilization of mRNA (Stern *et al*, 1989; Stern and Gruissem, 1987; Schuster and Gruissem, 1991). *In vitro* studies have shown that when 3' stem/loop structure were removed from their location in the 3' untranslated region of mRNAs either by deletion mutagenesis (Stern and Gruissem, 1989) or by endonucleolytic cleavage (Hsu-ching and Stern, 1991), the resulting RNAs were rapidly degraded. Direct evidence of stabilizing function of the 3' IR sequence were obtained from experiments in transformed *Chlamydomonas* chloroplasts, in which partial or complete deletion of the *atpB* 3' IR resulted in a 60 to 80% loss of mRNA (Stern *et al*, 1991).

The effect of translation on chloroplast mRNA turnover appears to be complex. The block of translation in leaf chloroplasts with linomycin, which inhibits an early step in polypeptide synthesis on 70S ribosomes prior to polysome assembly (Vasquez, 1979) reduces the decay of *psbA* and *lsu* mRNAs (Klaff and Gruissem, 1991). Chloramphenicol, which specifically inhibits the peptidyl-transferase activity of 70S ribosomes, accelerates the decay of *lsu* and *psbA*, but with different kinetics (Klaff and Gruissem, 1991). Within the general constraints of such inhibitor studies, these results suggests that translation of *psbA* and *lsu* mRNAs may be critical for initiating/facilitating their differential turnover. In addition, a polysome-free pool of *psbA* mRNA exists in higher plant chloroplasts, but the stability of this mRNA pool relative to the polysome bound *psbA* mRNA is not known (Klaff and Gruissem, 1991; Kelin *et al*, 1988; Minami *et al*, 1988). It is clear that ribosome association cannot be the only factor acting to increase the stability of these specific chloroplast mRNAs.

Taken together, the information presently available supports the view that the complex RNA processing found in chloroplasts is largely mediated and controlled by protein encoded in the nucleus. A large number of nuclear mutants in vascular plants have shown reduced levels of photosynthetic complexes (Metz and Miles, 1982; Miles, 1982; Gamble and Mullet, 1989; Kobayaschi *et al*, 1987; Taylor *et al*, 1986), but there are few published data on

chloroplast mRNA metabolism in such mutants (Barkan *et al*, 1986; Bruce and Malkin, 1991; Gamble and Mullet, 1989; Kobayaschi *et al*, 1987; Taylor *et al*, 1986). There is a class of proteins which has been shown to interact specifically with individual mRNA 3' ends (Stern *et al*, 1989). The function of these RNA-binding proteins has not been established, but it has been suggested that they are analogous to heterogeneous RNA binding protein particles and spliceosomes (Li and Sugiuara, 1990; Ye *et al*, 1991). Thus they may have a function in chloroplast intron splicing. Experiments using chloroplast-processing extracts in combination with RNA mobility shift and UV crosslinking assays have established that specific RNA-protein complexes form with the different 3'ends of *petB, lsu, psbA* (Stern *et al*, 1989), *rsp14*(Klaff and Gruissem, 1991) and *trnk* genes (Nickelsen and Link, 1989). These proteins were shown to interact with all mRNA 3' ends, although their binding constants differed for different RNAs (Schuster and Gruissem, 1991; Stern *et al*, 1989).

Translational control and post-translational regulation

Translation is an integral part of gene expression. Translational control uncouples the accumulation of a protein from the transcription of its gene.

Light control of translation has been shown during the light induced development of dark grown *Euglena gracilis*. Protein synthesis in isolated chloroplasts increased to about 100 fold, whereas specific mRNA increased no more than 3 times (Miller *et al*, 1983). In some cases, mRNAs have been found to be associated with polysomes without a corresponding accumulation of the proteins (Berry *et al*, 1988; Minami *et al*, 1988; Mullet *et al*, 1990). Other reports show high levels of chloroplast mRNAs with undetectable or low levels of the corresponding proteins (Berry *et al*, 1985; Berry *et al*, 1986).

An RNA binding protein of 47KD that interacts with the 5' end of *psbA* and *psbC* mRNAs has been isolated from *Chlamydomonas* chloroplasts. It is expected to function in translational control (Rochaiax, 1992). This protein accumulates to a similar level in dark and light grown cells and was found to remain bound to RNA when isolated from light-grown cells, suggesting that protein modification may be required for binding. Interaction of the 47KD

protein with *psbA* mRNA correlates closely with the *in vivo* translation of the PSB A protein.

Several photosynthetic proteins require noncovalent attachment of prosthetic groups such as chlorophylls, carotenoids, quinones, hemes and ions in order to function. Binding of these cofactors is often required for the protein to accumulate. This has been demonstrated for the nuclear-encoded (CAB) chlorophyll a/b binding light harvesting proteins, (Apel, 1979; Bennett, 1981), the chloroplast encoded chlorophyll apoproteins (Klein and Mullet, 1987; Sutton *et al*, 1987; Takabe *et al*, 1986; Vierling and Alberte, 1983) and plastocyanin (Merchant and Bogorad, 1986a; Merchant and Bogorad, 1986b).

Extensive studies have reported on the processing and light dependent degradation of the photosystem II PSB A protein (Mattoo *et al*, 1984; Ingali *et al* ,1989; Adir *et al*, 1990). The PSB A protein has been shown to be damaged by oxygen radicals during photosynthetic electron transport (Kyle *et al*, 1989) and is then degraded by a thylakoid membrane-associated protease (Sutton *et al*, 1987). The precise role of the proteolytic chloroplast enzymes and the control of their activities in the degradation of unassembled subunits, or turnover of subunits in membrane and soluble protein complexes, has not been established (Sutton *et al*, 1987).

Reversible phosphorylation of thylakoid membrane proteins has been known for more many years (Allen, 1983, 1992; Anderson, 1986; Bennett, 1983; Bennett, 1991; Staehelin and Arntzen, 1983) and has been most extensively studied for the light-harvesting chlorophyll a/b binding proteins (LHCP). Upon phosphorylation, the LHCP complex detaches from photosystem II and reattaches to photosystem I. This provides a mechanism for balancing the light harvesting capacity and electron flow between the two photosystems during energy spill overs. Dephosphorylation of LHCP is accomplished by thylakoid-bound phosphatases. Although most kinase and phosphatase activities are likely to be involved in the regulation of thylakoid membrane proteins and stromal enzymes, it cannot be excluded that they also target regulatory proteins that control transcription and translation of plastid proteins.

E. Control of protein biosynthesis

Some chloroplast proteins are synthesized inside the developing organelle, while many others are made in the cytoplasmic compartment of the cell. A traffic of nuclear-encoded polypeptides flows across the chloroplast envelope (reviewed by Ellis, 1981). Three multisubunit complexes have been shown to assemble in isolated chloroplasts: the light-harvesting chlorophyll a/b protein complex (Boschetti *et al*, 1990; Ellis, 1981), the chloroplast ATP-synthase complex (Nelson *et al*, 1980) and RUBISCO (Barraclough and Ellis, 1980; Chua *et al*, 1978a, b; Smith and Ellis, 1979). The latter two complexes contain both cytoplasmically-synthesized polypeptides and chloroplast-synthesized polypeptides.

Early studies using *in vivo* inhibitors have shown that SSU and CAB are the products of cytoplasmic protein synthesis (Barraclough and Ellis, 1975; Ellis, 1975; Ellis and Gray, 1986). The most striking features of such studies is that the transport is post-translational (Chua and Schimdt, 1978; Smith *et al*, 1979; Cline *et al*, 1985; Cashmore *et al*, 1985), i.e. it occurs after the polypeptide chains have been released from the ribosomes. This type of transport is thus different from that operating for secreted proteins where transport depends on concomitant protein synthesis (Blobel and Dobberstein, 1975; Schimdt *et al*, 1979). A similar post-translational mechanism has been found to operate for mitochondria (Bedbrook *et a*l, 1980; Zwizinski and Neupert, 1983; Ellis and Gray, 1986). The mechanism of post-translational transport involves specific interaction between the finished polypeptide precursor and the chloroplast envelope (Blobel, 1980; Dobberstein *et a*l, 1977). The sequence encoding the transit peptide is involved in this recognition process (Bedbrook *et al*, 1980; Smeekens *et al*, 1986).

F. Regulatory interactions of nuclear and chloroplast genomes

Chloroplast differentiation involves organ-specific, cell-specific (Whatley, 1979), and developmental stage-specific gene regulation (Mayfield *et al*, 1986). A key component of plastid development is the co-ordination of gene expression between the two different genomes i.e. chloroplast and

nucleus which are separated by several membranes (Susek and Chory, 1992). They are vastly different in complexity, structure, and gene organization. The mechanism governing the expression of nuclear and chloroplast genes are significantly different (Ellis, 1981; Benett *et al*, 1984).

Nuclear encoded photosynthetic genes expression

Numerous nuclear encoded photosynthetic genes have been cloned and characterized. A few of these are those for the small subunit of RUBISCO (*ssu*), ferredoxin NADP oxidoreductase, plastocyanin, CAB polypeptides of the light-harvesting complexes, the photosynthetic reaction centres and the chloroplastic ATPase (Langdale *et al*, 1988; Nagy *et al*, 1988; Flores and Tobin, 1986) .

All nuclear-encoded genes for chloroplast proteins investigated so far have mRNA with a 5' CAP ($m^{7'}$ $G^{5'}$ $ppp^{5'}$) and 3'-poly (A) sequence. They are translated on 80S ribosomes in the cytosol, where they direct the synthesis of pre-proteins that contain additional amino acids covalently attached to the N-terminus of the mature protein. These N-terminal extensions are similar to signal sequences that direct some proteins to the ER. They differ from the ER signals, however, in that they contain information for recognition and transport of the proteins by the chloroplast double envelope membranes (Ellis and Gaternby, 1984). This was first demonstrated for the *ssu* gene (Highfield and Ellis, 1978).

In pea, some of the nuclear genes for *ssu* contain two introns. The first of which seperates the 57-amino-acid transit peptide sequence from the region encoding the majority of the mature protein (Ellis *et al*, 1981). In other plants, the transit peptide varies from 40 to 60 amino acids. The transit peptide is recognized by a receptor on the chloroplast envelope and crosses the double membrane by post-translational mechanism. This requires ATP but not protein synthesis (Ellis *et al*, 1981). Once inside the envelope, the transit peptide is removed by soluble proteases in the stroma (Ellis and Gray, 1986; Mullet, 1985; Cline *et al*, 1985; Cline *et al*, 1991).

Studies of the transport and processing of *cab* proteins, which are located in the LHC II complex in the thylakoids, indicate that the precursor polypeptide may be incorporated into the membrane and processed

subsequently. This may also occur for some chloroplast-encoded thylakoid polypeptides (Apel, 1979; Klein and Mullet, 1987; Merchant and Bogorad, 1986a,b).

Transcription of two nuclear-encoded genes of chloroplast proteins, *cab* and *ssu* are regulated by light acting through phytochrome and a second blue-light receptor (Tobin and Silverthorne, 1986; Fluhr *et al*, 1986; Dedonder *et al*, 1993; Gallagher *et al*, 1985). Additional factors are responsible for organ-specific, cell-specific and developmental stage-specific modulation of transcriptional activity (Kuhlemeir *et al*, 1988; Langdale *et a*l, 1988; Aoyagi *et al*, 1988; Silverthorne and Tobin, 1984; Berry-Lowe and Maegher, 1985; Ernest *et al.*, 1987). *Cab* and *ssu* gene transcription has also been shown to exhibit circadian rhythm (Nagy *et al*, 1988, Busheva *et al* 1991; Giuliano *et al*, 1988) and are also influenced by cytokinins (Flores and Tobin, 1986). From this it can be concluded that the rate of transcription of *cab* and *ssu* genes in a given cell is dependent upon light, phytohormone and time of the day. Light can also affect gene expression at a post transcriptional level (Jenkins, 1991) including the level of transcript stability (Ernst *et al*, 1987; Wanner and Gruissem, 1991).

The role of phytochrome as the photoreceptor mediating the expression of many light responsive genes including *ssu* has been demonstrated in a variety of species (Tobin and Silverthorne, 1985; Fluhr and Chua, 1986; Clugstorn *et al*, 1990). In addition to phytochrome, blue /UV-A photoreceptor and UV-B photoreceptor(s) may be involved (Horwitz sand Gressel, 1986). An interaction between phytochrome and blue light receptor has been proposed by Thompson and White (1991). The interactions between these photoreceptors is thought to be complex; in pea, photoregulation of *ssu* genes depends on the developmental stage of the plant. In young etiolated pea seedlings, the light-induced changes in *ssu* expression are phytochrome mediated, and there is no evidence for action of photoreceptors. However, in mature leaves, a blue light receptor acts in concert with phytochrome (Fluhr and Chua, 1986; Sasaki *et al*, 1988; Clugston *et al*, 1990). Blue light receptors have been implicated in other species as well (Wehmeyer *et al*, 1990). Thus, it is clear that developmental and light cues must interact. Finally, inhibitor

and other studies show that the developmental state of the chloroplast influences *ssu* expression (Simpson *et al*, 1986; Thompson and White, 1991).

Similarly, individual members of the *cab* gene family in *Pisum sativum* (belongs to a mutigene family) have been shown to differ widely in fluence response (White *et al*, 1995). This group reported that at least two *cab* genes (cab-8 and AB 96) showed a very low fluence response to a single red light pulse. In contrast, two other *cab* genes (AB 80m and AB 66) fail to produce detectable transcripts following a single pulse of either red or blue light but were expressed in continuous red light. Thus, very low fluence responses and high irradiance responses occur in the same gene family.

Positional information determines the stage of a cell in the developmental program of a organ and the cell type that it will differentiate into (Kirk *et al*, 1978; Whatley, 1979). This position effect influences expression of nuclear-encoded genes. For example, a number of chloroplast enzymes belong to families of related isozymes that are differentially distributed in the plant. One good example is the glutamine synthetase of pea, which is encoded by four nuclear genes. One gene encodes the chloroplastic form, one a cytosolic form found in many organs and the other two genes code for the isoforms found in root nodules (Coruzzi *et al*, 1988). The gene coding for the chloroplastic form is expressed in leaves and its expression is induced by light acting in part through phytochrome (Tingelf *et al*, 1988). Thus, differential expression of the members of a nuclear gene family is an important component of the regulation of some genes coding for chloroplast proteins.

Chloroplast encoded gene expression

Monocistronic transcripts have been identified for at least eight genes: *psbA, atp H, lsu,* four tRNAs and the ribosomal protein *rps 16*. Polycistronic transcripts have been observed for six clusters: including *atpF-atpA, atpB-atpE,* the *r*RNAs genes, several tRNAs and some ribosomal proteins(reviewed by Whitfeld and Bottomly, 1983). As such, post-transcriptional mechanisms play an important role in regulating chloroplast-encoded gene expression. After transcription and processing, mature chloroplast mRNAs do not seem to be capped at the 5' end as are those in the cytosol, neither do they acquire a long poly-A tail at the 3'end (Malnoe *et al*, 1979; Poulsen, 1981).

Sequences in the untranslated region at the 3' end of some chloroplast genes have also provided interesting information (Deng *et al*, 1987). For example, there are two possible stem-loop structures in the gene for maize large subunit *lsu*, in the region where the mRNA ends, approximately 98 nucleotides down-stream from the termination codon for protein synthesis (Bogorad *et al*, 1983). This same region also contains two types of repeated sequence. It is believed that these features have significance for termination of transcription. Similar loops could occur at the 3'ends of other chloroplast mRNAs (Bohnert *et al*, 1982; Boyer *et al*, 1986). Related termination signals in the inverted repeats occurring just before the transcription stop sites have been found in a tobacco chloroplast genes (Shinozaki *et al*, 1986).

Interaction of the two genomes

An attractive hypothesis that has been proposed is that the products of nuclear genes are responsible for regulating each step of chloroplast gene expression. Gruiessem *et al* (1988) suggested that the proteins binding to the inverted repeats at 3'ends of chloroplast transcripts are responsible for controlling transcript turnover rates and these regulatory proteins are encoded in the nucleus. A number of nuclear mutations that interrupt specific processes in chloroplast biogenesis have been identified. These include mutations that disrupt the structural organization of thylakoid membranes (Martienssen *et al*, 1987), the assembly of multiprotein complexes (Leto *et al*, 1985; Rochaix and Erickson, 1988; Barkan *et al*, 1986), chloroplast transcription (Jensen *et al*, 1986), and chloroplast RNA processing and translation (Leto *et al*, 1985; Jensen *et al*, 1986; Kuchka *et al*, 1988; Taylor *et al*, 1988). In addition, a posttranslational mechanisms involving the co-ordinated accumulation of chloroplast proteins has been described. It has been shown that disproportionate transcription of *lsu* and *ssu* did not result in excess subunits because of rapid proteolysis of unassembled excess subunits (Nivision and Stocking, 1983; Schmidt and Mishkind, 1983; Berry *et al*, 1986, Rodermel *et al*, 1988).

A plastid factor has been hypothesized that can regulate nuclear gene expression by an unknown signal transduction pathway. This is based on a study of carotenoid-deficient leaves, where transcription of *ssu* and *cab* were shown to be reduced (Batschauer *et al*, 1986; Bugress and Taylor, 1988;

Giuliano and Scolink, 1988; Simpson *et al*, 1986). A similar reduction in *ssu* and *cab* mRNAs was observed in barley leaves treated with tagetitoxin, which inhibits chloroplast RNA polymerase (Sexton *et al*, 1990). In another study, maize mutants failed to accumulate *cab* mRNA when grown under high light intensity (Mayfield and Taylor, 1984; Mayfield *et al*, 1986). Together these data suggest that the generation of a putative chloroplast signal for maintenance of nuclear *ssu* and *cab* gene transcription is required for the transcription of chloroplast genes. Although the nature of the chloroplast signal is currently unknown, it is proposed to be generated through a metabolic intermediate of photosynthetic CO_2-fixation (Tonkyn *et al*, 1992).

Chloramphenicol treatment following chloroplast development showed no effect on nuclear gene expression (Oelmuller and Mohr, 1986). Similarly, heat induced block of chloroplast translation had no effect on nuclear gene expression (Bradbeer *et al* , 1979). Chloroplast protein synthesis is not critical to the signal transduction pathway once the chloroplast has developed.

G. Low temperature effects on expression of chloroplast proteins

There are few reports on the effect of low temperature on co-ordination of gene expression between the chloroplast and the nucleus. Both cold stress and acclimation appears to cause a loss of co-ordination. Hahn and Walbot (1989) noted a reduction of SSU polypeptide synthesis in cold-sensitive rice(*Oryza sativa* L. Texas variety). This reduction was greater than the reduction of LSU synthesis and this was correlated with low levels of *lsu* and *ssu* gene expression. However, this lack of co-ordination of LSU and SSU polypeptide synthesis did not affect the specific activity of RUBISCO. In another study by Meza-Basso *et al* (1986) showed the reduction of SSU polypeptide without a parallel decrease of LSU polypeptide during low temperature treatment in etiolated *Brassica napus* seedlings. In contrast with studies on accumulation pattern of LSU and SSU, showed that SSU was induced more than LSU in the cold-tolerant rice (Shakya and Agarwal, 1994). The variety of cold-tolerant rice used in their study is not mentioned.

Inhibition of chloroplast protein transport into chloroplasts by cold temperature has been shown (Grossman *et al*, 1980; Thieringer *et al*, 1991). This has been shown to be the result of low energy and not a result of altered membrane fluidity of the chloroplasts as previously believed (Leheny and Theg, 1994). As such, no functional adaptation takes place in the protein import machinery of chloroplasts in the cold-treated plants.

H. Additional information on specific genes studied in this thesis

Nuclear rRNA genes

Ribosomal RNA genes are found at one or a few loci in the genome. The genes are organized in long tandem arrays containing both gene and spacer sequences. The repeating unit in these arrays consists of one large transcription unit containing genes for the 18S, 5.8S and 25S rRNAs as well as spacer sequences that are removed during processing of the large primary transcript. Also included in the basic repeat are spacer sequences which are not part of the primary transcript and often called nontranscribed spacer or intergenic spacer sequences. The approximate number of nucleotides (which varies in different species) for 25S is 3580, 18S is 1926, 5.8S is 157 and 5S is 120 (Appels *et al*, 1980, Delseny *et al* , 1979; Ellis *et al*, 1984). The number of copies of both the major rRNA genes and 5S rRNA genes can vary widely among closely related species of plants, and even among different races or varieties within a species (MacKay *et al*, 1982; Maher *et al*, 1973; Siegl and Kolacz, 1983)

The major rRNA genes are transcribed by RNA polymerase I, a specialized form of RNA polymerase, specific for these genes. Active rRNA genes are found in the nucleolus where their transcripts are processed and assembled with ribosomal proteins (Grierson and Loeing, 1972; Coen and Dover, 1982; Reeder *et al*, 1983).

Genes for 5S ribosomal RNAs are also organized in tandem arrays, although they are located elsewhere in the genome away form the major rRNA gene arrays. They are transcribed by RNA polymerase III rather than polymerase I. As for the major rRNA genes, the arrays consist of alternating gene and spacer sequences (Moss *et al*, 1983; Ellis *et al*, 1984).

The cellular content of cytoplasmic rRNA is regulated by light in several plant species (Tobin and Silverthorne, 1985). Light-regulated changes in the rate of nuclear rRNA gene transcription have been demonstrated in pea (Gallagher and Ellis, 1982), *Lemna* (Silverthorne and Tobin, 1984) and barley (Mosinger *et al*, 1985).

Copy number has been shown to control rRNA transcription. Ribosomal RNA gene copy numbers can also vary substantially even among closely related genotypes within a species, indicating that needed supply of rRNA exceeds to the cytoplasmic ribosomal pool (Flavell *et al*, 1985).

It has been established in animal sytems that DNase I hypersensitive sites are frequently associated with actively transcribed genes (Elgin, 1981), including the ribosomal RNA genes of *Tetrahymena* (Palen and Cech, 1984), *Xenopus*(La Volpe *et al*, 1983) and Drosophila (Udvardy et al, 1984)). Similar light-regulated changes in DNase I hypersensitive sites in the rRNA genes of *Pisum sativum* has been reported (Kaufman *et al*, 1987). In *Triticum aestivum*, undermethylation is correlated with a DNase I-hypersensitive site (Thompson and Flavell, 1988).

Differences in the pattern and degree of methylation of ribosomal genes have been observed in many plants (Torres-Ruiz and Hemleben, 1994; Delseny *et al*, 1984; Siegel and Kolacz, 1983; Razin and Szcf, 1984; Olemedill *et al*, 1984). Hypomethylation is expected to lead to increased gene transcription (Scott *et al*, 1984; Flavell *et al*, 1986; Ellis *et al*, 1983; Watson *et al*, 1987; Bird *et al*, 1981).

Chloroplast rRNA genes

Chloroplast ribosomal RNA genes are arranged in an operon very similar to the ribosomal RNA operon of bacteria. The similar bacterial gene order, 16S-23S-4.5S-5S, is preserved in the chloroplast operons (Bedbrook *et al*, 1977; Delius and Koller, 1980; Gray and Hallick, 1979; Rochaix and Malnoe, 1978; Witfeld *et al*, 19178). The approximate sizes of the ribosomal genes, are 2810bp for 23S, 1490bp for 16S, 122bp for 5S and 65-103bp for 4.5S. The ribosomal operon in higher plants is located in the inverted repeat region of the DNA and there are consequently two copies per genome (Bedbrook *et al*, 1977; Bowman *et al*, 1981; Flur and Edelman, 1981; Jurgenson and Bourque,

1980; Rochax and Malnoe, 1978; Whitfeld *et al*, 1978). The spacer region between the 16S and 23S rRNA genes of chloroplast DNA contains genes for the tRNAs (Driesel *et al*, 1979; Malnoe and Rochax, 1978; Orozco *et al*, 1980).

Transcription of the chloroplast rRNA operon produces a single primary transcript containing the 16S, 23S 4.5S and 5S rRNAs, along with spacer tRNAs. This is then processed by a series of endonuclease cleavages to produce mature RNA products (Bohnert *et al*, 1982; Graf *et al*, 1980; Takaiwa and Sugiura, 1981).

There are no reports of chloroplast rRNA methylation with regards to their transcriptional activity. However, there is some evidence of tissue-specific methylation of plastid DNA from *Zea mays*, where methylation at *Hpa II* sites of the spacer region between the 16S and 23S rDNA from embyo, root tissue and endosperm have been observed (Gauley and Kossel, 1989).

RUBISCO

The gene for the large subunit of Rubisco, *lsu* maps in the large single copy region in approximately same position relative to rRNA operon (Bowman *et al*, 1981). Transcription of *lsu* varies from plant to plant. For example, transcription of spinach *lsu* is initiated 178-179 bp before the start of translation, whereas in maize this distance is only 63-64bp. Translational initiation site of *lsu* gene has shown that Shine-Dalgarno sequences are present just before the translation start site (Erion *et al*, 1981; Bedbrook *et al*, 1979).

Extensive work has shown that, in most higher plants, SSU is encoded by a gene family consisting of four to ten or more genes (reviewed by Dean *et al*, 1989). Further work in a number of species has shown that individual members of the gene family may be differently regulated, suggesting that a multigene family does not serve solely to amplify the product. For example, in the leaves of tomato plants all five *ssu* genes are expressed but at a different levels. (Sugita *et al*, 1987; Wanner and Gruissem, 1991; Dedonder *et al*, 1993). These variations are based on differences at both the transcriptional and posttranscriptional levels, variations in both DNA-protein interactions (Manzara et al, 1991) and mRNA stability (Wanner and Gruissem, 1991).

In all eukaryotes RUBISCO is an oligomer consisting of eight large subunits bound to eight small subunits. The synthesis of this oligomer requires a complex series of events involving both the nuclear and the chloroplastic genomes (Ellis and Gatenby, 1984; Ellis and Gray, 1986; Gutteridge and Gaetenby, 1987). The large subunit carries the catalytic site for both carboxylase and oxygenase activities, and is synthesized inside the chloroplast from *lsu* present in the chloroplast genome. The small subunit is required for enzymatic activity for some undefined reason, and is synthesized by cytoplasmic ribosomes as a higher molecular mass precursor from a family of *ssu* genes present in the nuclear genome. This precursor enters the chloroplast by an ATP-dependent protein transport mechanism; in the stromal compartment the aminoterminal extension is cleaved by a specific protease, and the mature small subunits assemble with the large subunits to produce the active holoenzyme (Ellis and Van der vies, 1988). The requirement of another chloroplast protein for the assembly of RUBISCO from its subunit in higher plants has been reported (Barraclough and Ellis, 1980). This binding protein is known as the RUBISCO binding protein. It is nuclear-encoded and its subunits are imported across the chloroplast envelope after synthesis on cytoplasmic ribosomes. The protein has also been suggested to mediate transport of large subunits within the chloroplast stroma from the site of synthesis to the site of assembly (Ellis and Van der vies, 1988).

psbA

The *psb A* gene for the 32kd thylakoid membrane protein *PsbA* is situated in the large single-copy region close to the end of the rRNA operon in the chloroplast genome. The direction of transcription of *psbA* is opposite to that of *lsu*. (Zurawski*et al*, 1982) In contrast to *lsu*, *psbA* does not contain the Shine-Dalgarno sequences before the translation initation site (Driesel *et al*, 1980). *psbA* have also been termed a photogene a gene whose transcripts increase appreciably in abundance during light-induced chloroplast development. Rapid light-dependent turnover of the 32kd protein in vivo was reported as early as two decades ago (Engleshman and Ellis, 1974 Mattoo *et al*, 1984). For example 32kd protein in pea exhibits a half life of 3hr at 400μmol m^{-2} s^{-1}(Aro *et al*, 1993).

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CHAPTER 2

Freezing tolerance of two different leaf ages in *Brassica napus* cv. Jet Neuf during low temperature acclimation

INTRODUCTION

Freezing tolerance is generally accepted to result from a combination of physiological and metabolic factors caused by altered gene expression (Levitt, 1980). Freezing tolerance is the ability of a plant to survive extracellular ice formation. In order for a plant to survive the cold stress, there are two major components that contribute to it's survival. They are the freezing tolerance in the non-acclimated state and freezing tolerance in the acclimated state. The ability to increase freezing tolerance, i.e. to survive at lower freezing temperatures after a period of low-temperature exposure is known as cold acclimation (Stone *et al,* 1993; Siminovitch and Briggs, 1953).

During cold acclimation, the increase in freezing tolerance can be measured by a number of methods, such as visible cell deterioration, changes in electrolyte leakage (Kulesza *et al*, 1986) or changes in luminescence (Brzostowicz and Barcikowska, 1987; Grabikwski, 1988). These tests are based on the following processes: when plants are cooled slowly, ice forms in the extracellular spaces (Siminovitch and Briggs, 1953). This results in osmotic contraction of the protoplast and causes disruption of the plasma membrane. The osmotic contraction of non-acclimated protoplasts results in endocytotic vesiculation of plasma membrane whereas in acclimated protoplasts, the osmotic contraction results in exocytotic extrusion of plasma membrane (Gordon-Kamm and Steponkus, 1984; Steponkus *et al*, 1983). In cells, plasmolysis results in exocytotic strands. If the strain is great enough the plasma membrane is damaged (Johnson-Flanagan and Singh, 1986; Singh *et al*, 1987). This damage leads to ion leakage from the tissue.

Electrolyte leakage is probably the most common test used. However, there remains some controversy whether ion leakage test is a good criterion for assessing freezing tolerance of plants, especially when dealing with different leaf ages (Hudson, 1961, Andrews and Morrison, 1992). It has been

stated that leaf discs fail to take into account changes in resistance which may occur during foliage maturation (Hudson, 1961). Also leaf tissue may be killed by freezing stress, but the plant survives by regrowth of the crown meristem (Andrews and Pomeroy, 1975, Gusta and Fowler, 1977). The other test that has been used for assessing freezing tolerance, which also indicates damage to the plasma membrane, is visual assessment which is based on the water-soaked appearance, chlorosis, necrosis, and loss of turgidity of the tissue (Kulesza *et al*, 1986; Hudson, 1961).

Growth is not uniform in plants. Cell division occurs primarily in the meristem, while subsequent growth is by cell expansion in regions closely associated with the meristem. There are some indications that growth controls the degree of freezing tolerance but the results are not definitive. Cox and Levitt (1969) showed a direct correlation between the growth rate and freezing tolerance but this correlation was not observed in young leaves. On the other hand, Macdowall (1974) reported a poor correlation between growth and freezing tolerance in spring and winter wheat. Since then, growth and freezing tolerance have been shown to be positively correlated in rye during low temperature acclimation (Uemura *et al*, 1989; Krol and Huner, 1985; Krol *et al*, 1984). For example, Uemura *et al* (1989) observed that in the young leaves the LT_{50} (-24^0C) was lower than the LT_{50} (-20^0C) of mature leaves. A possible explanation is that growth resulting from cell enlargement could be directly related to freezing tolerance and growth resulting from cell divison is inversely related to acclimation (Cox and Levitt 1969). Morphological (Cox and Levitt, 1969; Krol *et al* 1984; Krol and Huner, 1985) and biochemical changes (Krol *et al*, 1984; Krol and Huner, 1985) with freezing tolerance have been well documented but the role of cell divison has yet to be addressed. Further, the use of subjective terms such as older and younger may further hinder the interpretation of results.

It has also been stated that developmental stage rather than growth is related to freezing tolerance (Levitt, 1980) but evidence shows that this may not be true. For example, Cox and Levitt (1969) reported a high degree of freezing tolerance in young leaves relative to mature leaves. However, very young leaves were an exception. Further, the correlation between tissue age and freezing tolerance was very poor following a brief (24hr) acclimation

period. Thus, it remains unclear whether freezing tolerance is higher in young or older leaves.

The objective of this study was to assess freezing tolerance in mature (first fully expanded leaf) and young (fourth expanding leaf) leaves during four weeks of acclimation and to find an appropriate time period of acclimation, that would later permit examination of the changes in gene expression in mature and young leaves during low temperature acclimation. Therefore, the present study was designed to assess freezing tolerance by visual assessments on mature and young leaves of intact plants and electrolyte leakage tests on leaf discs. Cell division and developmental changes were studied in mature and young leaves in order to characterize the leaves with regard to developmental stage during cold acclimation. For the purpose of comparison, whole potted plants were frozen at the same time as the leaf discs and the injury on mature and young leaves from the intact plants were observed visually for fourteen days. Freezing tolerance of mature and young leaves were assessed at one, two, three and four weeks of acclimation.

MATERIALS AND METHODS

Growth conditions

Brassica napus cv. Jet Neuf was grown in controlled environment growth chambers, with light intensity of 350 µmol m^{-2} s^{-1} at 20^0C and a 16hr photoperiod. At the four leaf stage of development, the plants were moved to acclimating conditions of 4-5^0C with the same light intensity of 350 µmol m^{-2} s^{-1} and a 16hr photoperiod for four weeks. It has been observed that beyond four weeks of acclimation the plants grow into five leaf stage and the mature leaves begin to show signs of senescence. Therefore, the visual assessments and electrolyte leakage studies were done for up to four weeks.

Growth and development of mature and young leaves

Since growth at low temperature is slow, the non-acclimated plants reach the same developmental stage as that of acclimated plants at a different chronological age. In this study, the non-acclimated and acclimated plants were staged so that they were at the same developmental stage. These were

done on the basis of leaf lengths in the four leaf staged plants. Figure 2a and 2b show the days where both non-acclimated mature and young leaves reached the same developmental stage as that of acclimated mature and young leaves at each weeks of study.

Leaf disc freezing protocol

Throughout the four weeks of acclimation, for the leaf disc experiments, non-acclimated and acclimated plants were staged so that they were at the same developmental stage. From ten plants, leaf discs (1cm in diameter) were sampled from different regions avoiding the mid vein region of both mature (the first fully expanded leaves) and young leaves (fourth expanding leaves). The leaf discs in replicates of four, (each petri dish contained two leaf discs, one from mature and the other from young) were blotted on the wet filter papers (Whatman cellulose filter paper, 2.1 cm) in petri dishes (60 X 15mm standard, Fisher). One of the filter discs was notched to distinguish one from another and deionized water was used to soak the filter discs. After equilibration at 0^0C for 30 minutes, four petri dishes were removed (this represented the 0^0C sample) and moved to 4^0C and the temperature was dropped to -2.5^0C over one hour. The samples were then nucleated by placing a chip of dry ice on the filter paper, and then equilibrated for another 8hr. Thereafter, the rate of cooling was 2^0C/hour. Samples were removed at -2.5^0C, -5^0C, -7.5^0C, -10^0C, -12.5^0C, -15^0C and -17.5^0C intervals and were thawed at 4^0C overnight, prior to determining electrolyte leakage.

Electrolyte leakage test

The method of Hodgson (1964) was used with the following modification. The leaf disc and filter paper were transferred to 50ml Falcon tubes containing 10 ml of deionized water and were shaken for 24 hours at room temperature in light. The conductivity was then measured with a CDM 83 conductivity meter (Radiometer, Copenhagen, Bach-Simpson ltd., London, Ontario). The tissue and solution in the tubes were heated to boiling (3 min) in a microwave oven. The tubes were allowed to cool to room temperature for one hour and the specific conductivity of the boiled samples was remeasured. Electrolyte leakage due to freeze-thaw stress was expressed as %

of the total and the temperature of 50% leakage used as an indicator of lethal damage (Sukurmaran and Weiser, 1972).

Δ ion leakage = [Leakage caused by freezing / Leakage after boiling (total)] X 100.

Determination of RFT values

Relative freezing tolerance (RFT) was determined according to the method of Stone *et al,* (1993). Determination of RFT values were based on ion leakage measurements of leaf discs (mean of four replicates) after freezing at specific temperatures. The temperature corresponding to 50% ion leakage was taken from the midpoint of the maximum and minimum ion leakage. This was determined at each week in mature and young leaves of both acclimated and non-acclimated plants.

Whole plant freeze protocol

Plants were staged throughout acclimation, so that all tests were completed using plant material at the same developmental stage. At the four leaf stage, plants were placed in a programmed freezer and equilibrated at 0^0C for 30 minutes (in two replicates i.e. two plants/pot). At this temperature two replicates of non-acclimated and acclimated plants were removed, and transferred to 4^0C for 7 days. This represented the 0^0C treatment. The temperature was dropped to -2.5^0C over one hour. The plants were then sprayed with distilled water and nucleated with a fine dust of ice. The temperature was held at -2.5^0C for 8 hr to ensure plant equilibration. Thereafter the cooling rate was 2^0C/hr. Two replicates of both control and acclimated plants were removed at -5^0C, -10^0C, -15^0C and -17.5^0C intervals, thawed at 4^0C and grown for seven days at 4^0C. After seven days at 4^0C, the plants were moved to the green house for another seven days.

Visual assessment of mature and young leaves.

After freeze testing, mature and young leaves of intact plants were assessed for seven days at 4^0C and another seven days in the greenhouse. Qualitative assessments were made for loss of turgidity, chlorosis, necro is

and water soaked appearance. Leaves were evaluated on a scale where six denoted a healthy leaf and one indicated a dead leaf.

Measurement of in vivo DNA synthesis

^{3}H-thymidine (Amersham) (92.5 X 10^{10} Bq mol^{-1}) was used to label DNA. Small areas on the upper surface of mature and young leaves were washed with 70% ethanol prior to spotting with 18.5 X 10^{4} Bq ^{3}H-thymidine. Plants were returned to acclimating conditions for 24hrs.Thereafter, each sample was ground in a small volume of CTAB buffer [100MM Tris HCl pH 8, 1.4M NaCl, 20mM EDTA, 2% CTAB, 0.2% β-mercapatoethanol (added just before grinding)] with a mortar and pestle and spotted onto GF/C filter discs (Whatman). Total counts were determined on one filter while the incorporated counts were determined on the other filter by precipitating with 10% TCA for 30 minutes, followed by 5% TCA for another 15 minutes and finally in 95% ethanol for 15 minutes.

Leaf area and weight

Leaf area was calculated using a LI-3100 area meter (Li-Cor Inc., Lincoln, Nebraska). Fresh leaves were weighed, dried at 60^{0}C for 48hr and reweighed.

RESULTS

Relative freezing tolerance of mature and young leaves

Acclimated mature leaves showed an increase in relative freezing tolerance within one week of acclimation (Table 2.1). Relative freezing tolerance measurements showed that the increased acquired freezing tolerance was maximum at three weeks. The relative freezing tolerance of mature leaves changed from -4^{0}C to -11^{0}C over 3 weeks of acclimation. At four weeks of acclimation, mature leaves showed a loss of the acquired relative freezing tolerance. The relative freezing tolerance in the non-acclimated mature leaves changed from -4.0^{0}C to -8.0^{0}C over four weeks .

Acclimated young leaves showed increased freezing tolerance by one week of acclimation, with maximum acquired tolerance at two weeks (Table 2.1). The relative freezing tolerance remained steady between the second and fourth week. The RFT values in young leaves changed from -5^{0}C to -13.5^{0}C

over 3 weeks of acclimation. In contrast, in non-acclimated young leaves the relative freezing tolerance changed from -5^{0}C to -8.5^{0}C.

In the non-acclimated state mature and young leaves showed similar relative freezing tolerance over the four weeks. In the acclimated state, the difference between the relative freezing tolerance of mature and young leaves was the least at three weeks. The acquired freezing tolerance was greater in young leaves than in the mature leaves.

Visual assessments of mature and young leaves

Visual assessments showed that acclimated mature leaves acquired maximum tolerance of -10^{0}C at three weeks (Fig 2c) and began to loose tolerance thereafter. Non-acclimated mature leaves showed a gradual increase in tolerance but did not display the same tolerance achieved in acclimated mature leaves (-5^{0}C versus -10^{0}C).

Visual assessments of young leaves showed that there was a steady increase in freezing tolerance with acclimation time (Fig. 2d). Acclimated young leaves showed maximum acquired freezing tolerance at three weeks of acclimation. Non-acclimated young leaves showed an increase in freezing tolerance but did not display the same tolerance achieved in acclimated young leaves.

Between the two leaves, acclimated young leaves showed a greater tolerance to freeze-test temperatures than acclimated mature leaves. The differences between the acquired tolerance of acclimated mature and young leaves was the least at 3 weeks.

Cell division and developmental changes in the acclimated leaves

The relationship between acclimation, developmental changes and cell division was elucidated by measuring fresh weight, dry weight and leaf area and quantifying the incorporation of ^{3}H-thymidine in mature and young leaves over the acclimation period. Young leaves underwent a greater change in fresh weight, dry weight and leaf area over the four week period, relative to mature leaves (Fig 2e and 2e-1 inset). Leaves of both ages showed cell division, as indicated by ^{3}H-thymidine incorporation (Table 2.2). Over the first two weeks of acclimation total incorporation was 10 times more in young

leaves in comparison to the mature leaves. The rate of cell division decreased in the young leaves in the third and fourth week, but was still at least double that of the mature leaves.

DISCUSSION

The primary focus of the present study was to assess freezing tolerance in the two leaf ages and to find a time period of acclimation where the two leaves would be comparable in their freezing tolerance, thus permitting further studies on changes in gene expression of the two leaf ages in *Brassica napus* during low temperature acclimation.

LT_{50}s based on electrolyte leakage data are known to vary with the type of tissue and time of acclimation. In this study, the relevance of this assessment method was determined for both leaves of acclimated and non-acclimated plants and acclimation period by comparing the results with those from the visual assessment of injury.

The results of the visual assessments and electrolyte leakage tests were comparable. In the present study, data shows that in the non-acclimated state, mature and young leaves displayed similar relative freezing tolerance at each week of acclimation. However, with acclimation, the mature and young leaves differed in their acquired freezing tolerance. From both tests, it was observed that both mature and young leaves acclimated during low temperature exposure as they displayed an increase in tolerance to freeze-test temperatures. Results from the visual tests and the ion leakage data indicated that mature leaves showed maximum freezing tolerance at three weeks of acclimation. After four weeks of acclimation, both tests indicated that there was a loss of acclimation potential in acclimated mature leaves. Both tests showed that young leaves were more tolerant to freeze-test temperatures than mature leaves. The smallest difference of the acquired freezing tolerance between mature and young leaves was at three weeks.

Changes in non-acclimated freezing tolerance with development were noted in the present study. Both tests revealed large increases in tolerance in both leaf ages over the study period but no difference between the leaf ages. This may suggest that developmental stage has a role in freezing tolerance.

Similar trends in the increased freezing tolerance with time have been observed in non-acclimated winter rye leaves (Krol *et al*, 1984).

An inverse relationship between freezing tolerance and leaf age has been stated (Levitt, 1980). However, this was poorly substantiated by Cox and Levitt (1969). In the present study, freezing tolerance, whether determined by the electrolyte leakage test or by visual assessment, was greater in the young leaves at all stages of acclimation. This is in agreement with the results of Uemura *et al* (1989) in which they observed maximum freezing tolerance in young rye leaves. As such, the age of leaves should be taken into account when assessing freezing tolerance of a tissue.

Many workers dispute that morphological changes can bring about increased freezing tolerance (Cox and Levitt, 1969; Huner, 1985; Krol *et al*, 1984; Weiser, 1970), although some changes, such as increases in dry matter, are known to render the tissue more freezing tolerant. In the present study, it was found that both fresh and dry weight increased in the young leaves. Changes in fresh and dry weight could be a result of newly synthesized cytoplasmic material (Krol *et al*, 1984).

Changes in the leaf area, fresh weight or dry weight probably accompanied acclimation in young leaves. This agrees with the response in acclimating rye leaves, despite the different tissue types studied and the acclimation time utilized (Krol *et al*, 1984). In an attempt to reduce the confounding effects of morphological change, the present study used a short acclimation period (up to four weeks), whereas Krol *et al*, (1984) focused their research on the growth kinetics and used an acclimation period of seven to ten weeks. It is interesting to note that even though Krol *et al* (1984) noticed the growth response of individual leaves differed but did not asses freezing tolerance of indiviual leaves but rather used the uppermost fully expanded leaves to assess freezing tolerance. They also stated that leaf development influences its growth response to an altered environment.

Cox and Levitt (1969) stated that young leaves did not show a direct relationship between the degree of freezing tolerance attained and growth rate of the leaves. It was observed that prolonged acclimation led to high degree of tolerance of young leaves in the absence of rapid growth. In contrast, during a

24hr acclimation period young leaves showed the smallest increase in tolerance and largest relative growth rate. Therefore, they suggested that growth resulting from cell division was inversely related to freezing tolerance. The results from the present study do not support this claim as young leaves, had high rates of cell division and attained maximum tolerance. Further, the rate of cell division decreased between weeks three and four, in both leaves, while freezing tolerance decreased in the mature leaves and remained constant in the young leaves.

The main objective of this study was to find an appropriate acclimation period for the two leaf ages so that further studies on changes in gene expression on the two leaf ages could be examined during low temperature acclimation. Three weeks of acclimation was selected for further study as there was the greatest difference in freezing tolerance between acclimated and non-acclimated leaves and freezing tolerance was maximal in both leaf ages. This is the first study where freezing tolerance of two different leaf ages have been shown to change with development during low temperature acclimation. Acclimation in mature leaves was associated with higher rates of dry matter accumulation while acclimation in young leaves was associated with higher rates of cell division.

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Table 2.1 Relative freezing tolerance (RFT) values in mature and young leaves of non-acclimated and acclimated leaves. The relative freezing tolerance was calculated from plots of % ion leakage from 4 separate determinations. The non-acclimated were developmentally staged to be equivalent to the acclimated leaves (see figure 2a and 2b). Therefore the non-acclimated and acclimated leaves used were of different chronological age. Maximum SE $\pm$0.47.

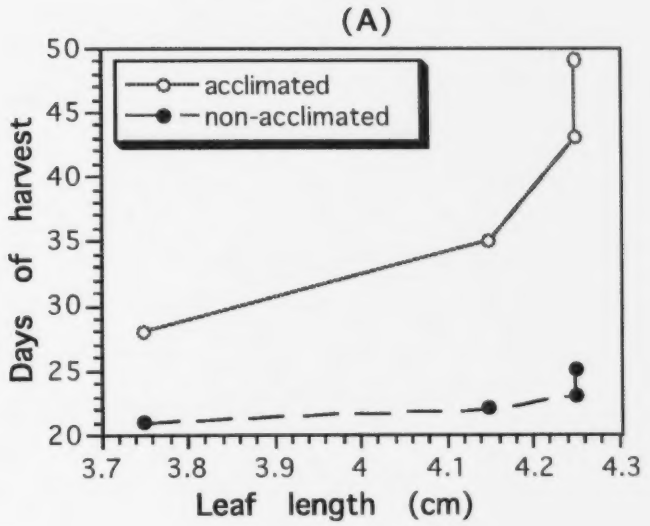
Days of harvest		Mature leaves		Young leaves	
20° C	**4° C**	**20° C**	**4° C**	**20° C**	**4° C**
21	27	-4° C	-6.5° C	-5° C	-9.5° C
22	34	-7.5° C	-9° C	-8° C	-13.5° C
23	42	-6.5° C	-11° C	-6.5° C	-13° C
25	48	-8° C	-9.5° C	-8.5° C	-13° C

Table 2.2 Percent ^{3}H-Thymidine incorporation during 24hrs labelling in mature and young leaves during low temperature acclimation. Values are the incorporation label divided by the total label in the tissue. Values are the means from 3 different experiments. Maximum SE$\pm$.028

Weeks of acclimation	Mature leaves	Young leaves
0	1%	10%
2	0.9%	9%
3	1.4%	3.7%
4	1.3%	4%

Figure 2 (a) Length of non-acclimated and acclimated mature leaves (cm) in four leaf staged *Brassica napus* plants during four weeks of cold acclimation. Values are the means from 10 plants. Maximum SE$\pm$0.34.

Figure 2 (b) Length of non-acclimated and acclimated young leaves (cm) in four leaf staged *Brassica napus* plants during four weeks of cold acclimation.Values are the means from 10 plants. Maximum SE$\pm$.029.


(A)
acclimated
non-acclimated
Days of harvest
50
45
40
35
30
25
20
3.7
3.8
3.9
4
4.1
4.2
4.3
Leaf length (cm)

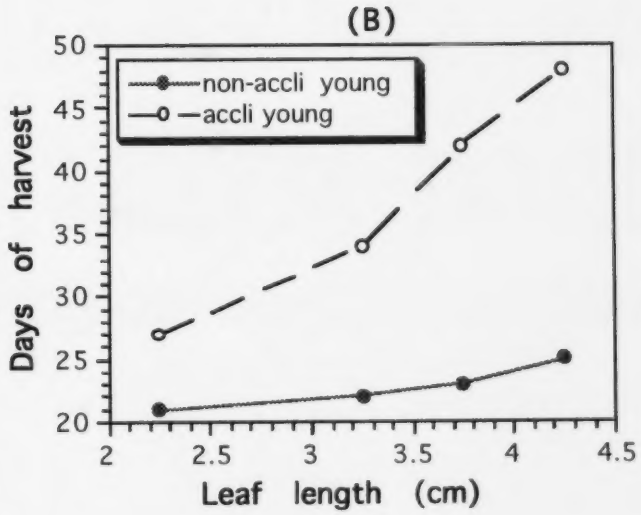
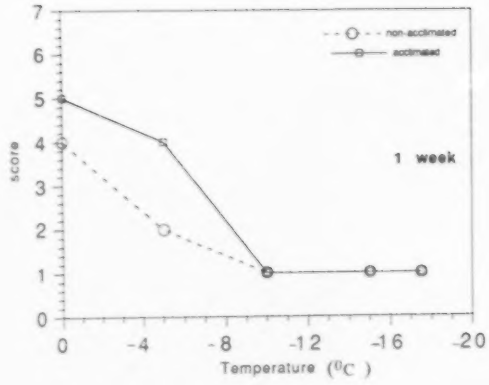
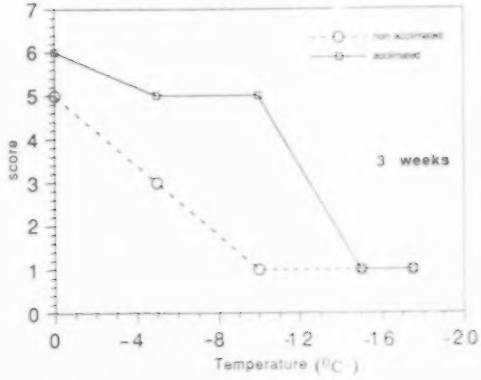
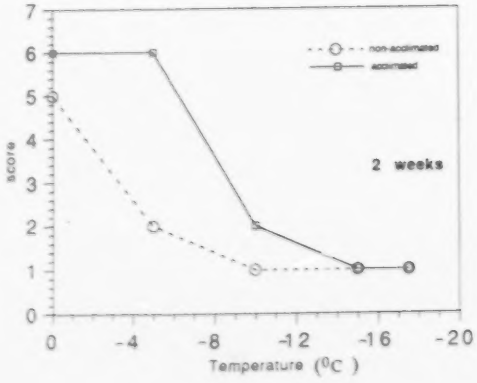

(B)
non-accli young
accli young
Days of harvest
50
45
40
35
30
25
20
2
2.5
3
3.5
4
4.5
Leaf length (cm)

Figure 2c Visual assessments on day fourteen of mature leaves from intact plants of acclimated and non-acclimated plants after freeze-testing. Values represent, the mean of 2 separate experiments, each with 2 replicates.


non-acclimated
acclimated
1 week
score
Temperature (°C)


non-acclimated
acclimated
3 weeks
score
Temperature (°C)


non-acclimated
acclimated
2 weeks
score
Temperature (°C)

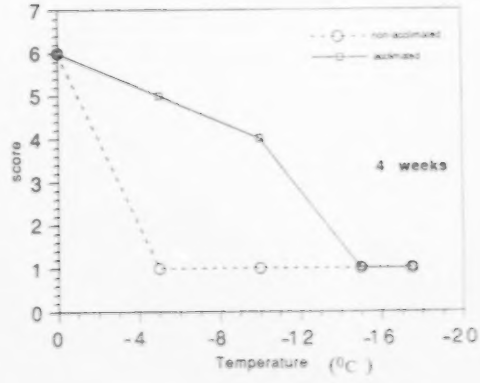
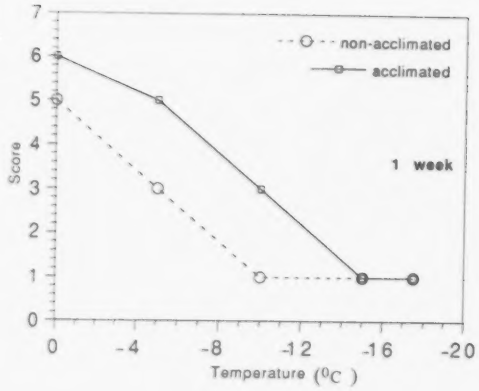
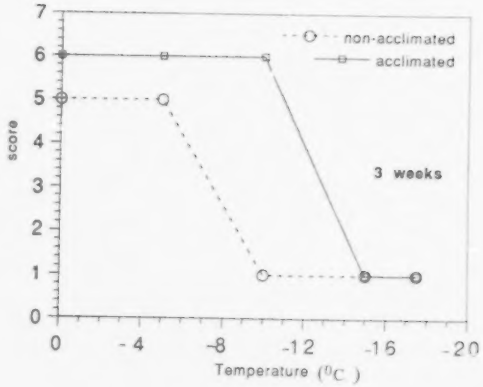
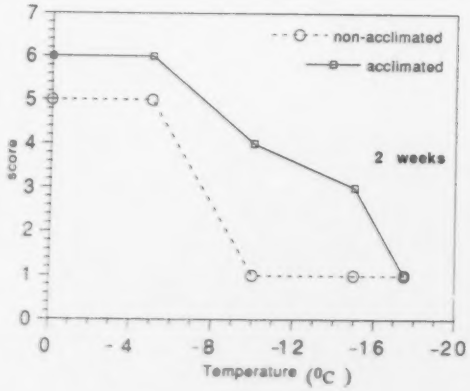

non-acclimated
acclimated
4 weeks
score
Temperature (°C)

Figure 2d Visual assessments on day fourteen of young leaves from intact plants of acclimated and non-acclimated plants after freeze-testing. Values represent, the mean of 2 separate experiments, each with 2 replicates.


non-acclimated
acclimated
1 week
Score
Temperature (°C)


non-acclimated
acclimated
3 weeks
score
Temperature (°C)


non-acclimated
acclimated
2 weeks
score
Temperature (°C)

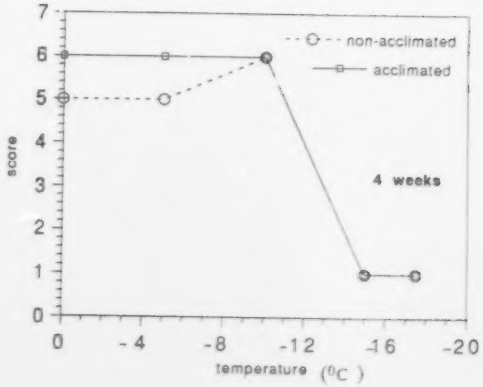

non-acclimated
acclimated
4 weeks
score
temperature (°C)

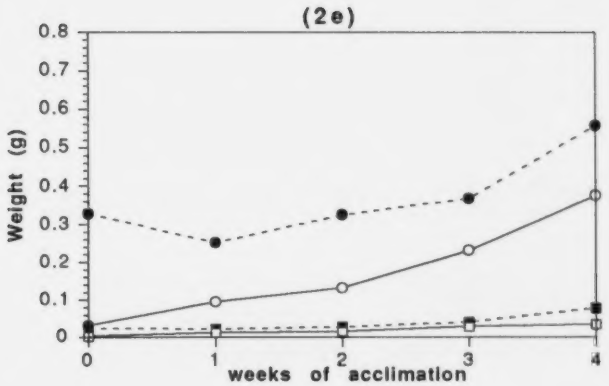
Figure 2e Changes in fresh weight and dry weight (g) during cold acclimation. Values are the means of 10 determination Maximum SE ±.026.

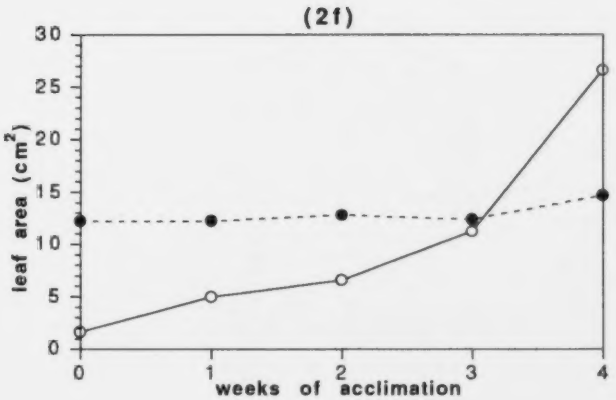
● Fresh weight of mature leaf o Fresh weight of young leaf

■ Dry weight of mature leaf □Dry weight of young leaf

Figure 2f Changes in leaf area during acclimation. Values represent the means of 10 determination. Maximum SE±.031

● Mature leaf o Young leaf


(2e)
Weight (g)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
weeks of acclimation


(2f)
leaf area (cm²)
30
25
20
15
10
5
0
0
1
2
3
4
weeks of acclimation

CHAPTER 3

Expression of rRNA and the possible role of rDNA methylation during low temperature acclimation in *Brassica napus* cv. Jet Neuf

INTRODUCTION

During the induction of freezing tolerance at low temperature, increases in RNA content reflects the various changes in the protein synthesizing machinery (reviewed by Johnson-Flanagan and Singh, 1988). This could range from overall increases in ribosomal RNA, mRNA and tRNA to a combination of increases and decreases of RNA classes.

Increases in nuclear rRNA transcription during the induction of freezing tolerance have been reported by several workers (Zvereva and Turnova, 1985; Sarhan and Chevrier, 1985; Paldi and Devay, 1977; Sarhan and D'Aoust, 1975). For example, during cold acclimation in boxwood, a 50% increase in the rate of rRNA synthesis was reported by Gusta and Weiser, (1972). Similarly, in winter wheat, increased rates of rRNA synthesis have been observed while it was not observed in spring wheat cultivar (Paldi and Devay, 1977). Further research on rRNA synthesis during cold acclimation and breakdown of rRNA during deacclimation in winter wheat demonstrated the presence of different rRNA size classes, one which was synthesized at low temperature and decomposed at higher temperature, while the other was synthesized at low temperature and stable at that temperature (Paldi and Devay, 1977).

Considerable attention has been given to increased transcription of nuclear rRNA during cold acclimation (Paldi and Devay, 1977; Gusta and Weiser, 1972; Sarhan and Chevrier, 1985; Zvereva and Turnova, 1985). While the requirement for increased rRNA synthesis is essential (Zeverva and Turnova, 1985) for attaining cold tolerance and for maintaining growth at low temperature, little is known about the molecular mechanisms controlling the increase, nor is it clear whether increased transcription is responsible for increased nuclear rRNA levels. Further, workers have focussed their work on

nuclear rRNA, and little attention has been given to chloroplast rRNA, even though the chloroplast also synthesizes its own protein (Mayfield, 1990).

The rate of transcription of a gene can be regulated by the accessibility of the gene to RNA polymerase. This can be altered by gene methylation (Cedar, 1988; Kaufman *et al*, 1987; Flavell *et al*, 1986; Ellis *et al*, 1983; Bird *et al*, 1981). Ribosomal genes have been reported to be methylated in higher plants, such as in *Cucurbita pepo, Cucumis sativus, Cucumis pepo, Brassica rapa*, and *Nicotiana glauca* (Scott *et al*, 1984; Uchimiya *et al*, 1982). Changes in rRNA expression in relation to methylation have been shown by Kauffman *et al*, (1987). They reported that there were two groups of rDNA genes. One set was constitutively expressed while the other was only expressed during light induction. It was found that this induction was caused by hypomethylation of the promoter region on the second set of genes.

The hypothesis proposed for this study was that chloroplast and nuclear rRNA expression is altered during low temperature acclimation and/or development in a co-ordinate manner. Further, increased expression is correlated with increased freezing tolerance. Finally, changes in chloroplast and nuclear rRNA expression are controlled by methylation of the ribosomal genes. The objective of this study was to examine rRNA changes in mature and young leaves of *Brassica napus* cv. Jet Neuf during low temperature acclimation as previous studies show that with increased freezing tolerance there is increased rRNA expression. A previous study (Chapter 2) determined that mature and young leaves acclimated for three weeks were the most suitable tissue for the present study. Therefore, both nuclear and chloroplastic rRNAs were examined in mature and young leaves during three weeks of cold acclimation. The role of site specific methylation was examined as a possible control of gene expression.

MATERIALS AND METHODS

Plant growth conditions

Brassica napus cv. Jet Neuf seeds were germinated in a controlled growth chamber at 20^0C (d/n), with a 16 hr photoperiod, and light intensity of 350µmol m^{-2} s^{-1}. At the four leaf stage, plants were moved to 4-5^0C (d/n) with the same photoperiod and light intensity in a controlled growth

chamber to acclimate for three weeks. Growth under low temperature acclimating conditions was slow, therefore, acclimated plants reached the same developmental stage as non-acclimated plants at a different chronological age. As such, mature and young leaves from the non-acclimated plants were harvested at 23-24 days and at 42-43 days from the acclimated plants. Tissue was collected at the same time in the light cycle. The leaves were freeze dried and stored at -80^{0}C prior to extraction of RNA and DNA.

Total RNA extraction and quantification

RNA was extracted according to the methods of MacDonald *et al*, (1987). 10g of leaf material was homogenized in grinding buffer [4M guanidinium thiocyanate, 50mM Tris-HCl pH7.6, 2% N-lauryl sarcosine Na salt, 1% β mercaptoethanol] in a 2:1 ratio on ice. The homogenate was centrifuged at 7^{0}C for 10 minutes at 2310Xg (SS34 rotor). The supernatant was made up to 2.4M with 6M CsCl, and was layered over 9 ml of 5.7M CsCl in 0.1M EDTA and centrifuged at 100,000Xg (SW27 rotor) for 18 hr at 7^{0}C. The resulting RNA pellet was resuspended in DEPC treated water and partitioned with phenol-chloroform four times until the interface was clean. The sample was precipitated overnight in 0.1 volume of 3M sodium acetate (pH 5.2) and 2.2 volume of ethanol (95%) and centrifuged at 13,000 rpm in a bench micro centrifuge for 10 minutes. After centrifugation the RNA pellet was digested with DNase I (RNase free; Boehringer Mannheim) prior to quantification: RNA was dissolved in 0.02 M Tris (pH 7.4) and 1 mM $MgCl_2$, 10 units of DNase I was added. The mixture was incubated at 37^{0}C for 30 minutes. An equal volume of grinding buffer was added and the sample was extracted with phenol-chloroform four times at room temperature then precipitated with 0.1 volume of 3M sodium acetate and 2.2 volume of 95% ethanol overnight at -20^{0}C and centrifuged at 13,000 rpm in a bench micro centrifuge for 10 minutes. The resulting RNA pellet was dissolved in DEPC treated water, checked for contamination of DNA by running a small amount (5μg) on a test gel (0.8% TAE agarose gel) and the RNA concentration was determined spectrophotometrically (Varian Cary 219) at 260 and 280 nm.

Isolation of total plant DNA

Total genomic DNA was isolated according to the methods of Taylor *et al,* (1993) with modification. Leaf material (1g) was homogenized in 15ml CTAB buffer [100mM Tris HCl pH8, 1.4M NaCl, 20mM EDTA, 2% CTAB, 0.2% β- mercaptoethanol (added just before grinding)] at room temperature and transferred to 50ml Corex tubes. Following incubation at 60^0C for 30 minutes, the sample was extracted with 15ml chloroform:isoamyl alcohol (24:1) and centrifuged at 2330Xg (SS 34) for 10 minutes. The chloroform:isoamyl alcohol extraction was repeated 2-3 times until the interface was clean. 0.6 volumes of 2-propanol was added to the aqueous layer. The DNA was spooled out of the tube with the aid of a sterile glass hook and placed in a microfuge tube, washed in 70% ethanol at room temperature for 20 minutes, and centrifuged at 1000rpm in a bench micro centrifuge, and air dried. Following air drying, the DNA pellet was dissolved in TE buffer (pH 8) [10 mM Tris pH 8 and 1mM EDTA] to which RNase (10 units, DNase free, Boehringer Mannheim) was added, and then incubated at 37^0C for 30 minutes. Following incubation, the sample was chloroform extracted and 0.6 volumes of 2-propanol was added to the aqueous phase. DNA was pelleted at 12,000rpm in a bench micro centrifuge at 4^0C and dissolved in 1ml TE buffer (pH 8).

Isolation of chloroplasts

Chloroplast isolation was done according to the method of Herrmann, (1982). Mature and young leaves (20g) were harvested on ice, then homogenized in 100ml ice cold grinding medium [0.3M sorbitol, 50 mM HEPES/KOH buffer pH 8.3, 2mM $MgCl_2$, 1mM $MnCl_2$] in a Waring blender for 2 seconds. The homogenate was filtered through four layers of cheese cloth and collected on ice, then centrifuged for 4 minutes at 2500Xg (SS 34) at 4^0C. The supernatant was discarded and the crude chloroplast pellet was resuspended in 1ml of grinding medium. The chloroplast suspension was carefully layered on a Percoll gradient and centrifuged at 9000Xg at 4^0C for 15 minutes in a swinging bucket rotor (SW 27). The lower intact chloroplast band was carefully removed and diluted in 1ml of grinding medium. The Percoll gradient (35ml) was prepared by pipetting 50% PBF-Percoll [140ml Percol, 4.2 g PEG $_{8000}$, 0.14g BSA and 1.4g Ficol $_{400}$] , 0.35 M sorbitol, 50mM HEPES/KOH buffer pH 8.3, 1mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM Na_2EDTA

into a 50-ml polycarbonate tubes and centrifuging at 40,000Xg (Ti 50) for 40 minutes at 4^0C. After centrifugation, the tubes were stored at 4^0C.

Isolation of chloroplast DNA

Isolation of chloroplast DNA was done according to the methods of Herrmann, (1982). The chloroplast sample was made up to 2.7 ml with wash buffer [350 mM sorbitol, 50 mM Tris-HCL (pH 8.0), 20 mM EDTA] and then 0.3 ml of 10 mg/ml proteinase K solution was added. The solution was mixed gently by inversion and incubated at room temperature for 15 minutes. After incubation, 0.3ml of 20% sarkosyl (N-lauryl sarcosine Na salt) was added and mixed gently by inversion and then incubated at room temperature for 50 minutes. One ml of 0.672g/ml CsCl stock solution [26.88g CsCl adjusted to final volume of 40 ml with 50 mM Tris-HCL(pH-8), 20 mM EDTA] was mixed by gentle inversion and allowed to stand for 1 hour at 4^0C. The suspension was then centrifuged at 12,000Xg for 20 minutes and the supernatant containing DNA was carefully decanted into a 10-ml graduated cylinder. The volume was adjusted to 4.8 ml per gradient with Tris-EDTA buffer. For each gradient, 6.7 ml of the 1.24g/ml CsCl stock solution [248g CsCl adjusted to final volume of 200 ml with 50 mM Tris-HCl (pH-8), 20 mM EDTA] and 0.3 ml of a 10mg/ml ethidium bromide solution was added to 4.8ml of DNA solution. The solution was slowly inverted to mix and then centrifuged at 150,000Xg (Ti 50) for 40 hours at 20^0C. After centrifugation, the DNA bands were visualized with a 360 nm UV source. The upper band was carefully removed and extracted with an equal volume of TE buffer-saturated isopropanol to remove ethidium bromide. This was done several times until the pink color was absent in the upper phase. The upper phase was discarded and the lower phase was precipitated overnight with 1 volume of TE buffer and 2 volumes of 95% ethanol at -20^0C. A second ethanol precipitation was performed to remove the residual CsCl. The final DNA pellet was resuspended in TE buffer and the concentration was determined spectrophotometrically (Varian Cary 219) at 260 nm and 280 nm.

Extraction and quantification of DNA

The Volkin and Cohn (1964) method was used for extraction of nucleic acid and quantification of DNA in the sample. The leaf homogenate was

made 0.4N with respect to perchloric acid and was centrifuged at 3000Xg at 4^0C for 15 minutes. The supernatant was discarded and the pellet was washed twice with 10ml of 0.4 N perchloric acid and twice with 10ml of 95% alcohol. The lipids were extracted by three washes with 10ml of 3:1 ethanol:ether (v/v) at 40-50^0C. After the lipid extraction the pellet was washed by suspension in 5 volumes of 10% NaCl (pH 7) and the mixture was heated for 1hr at 100^0C to extract nucleic acids. After centrifugation at 3000Xg (SS34) for 10 minutes, the supernatant was filtered through glass wool, and the extraction repeated on the pellet with 3 volumes of 10% NaCl solution and 30 minutes of heating at 100^0C. The NaCl extract was treated with 3 volumes of cold 95% ethanol and was allowed to stand at 0^0C overnight to precipitate nucleic acids. The nucleic acids were pelleted by centrifugation at 1610Xg (SS34) for 20 minutes at 4^0C. The pellet was resuspended in 1ml TE (pH 8). DNA was quantified by the diphenylamine reaction for deoxypentose (Volkin and Cohn 1964), as follows. The diphenylamine reagent was prepared by dissolving 1.0g of diphenylamine in 98ml of redistilled glacial acetic acid and 2ml of concentrated H_2SO_4. The reagent was kept at 4^0C. The nucleic acid sample (1ml) was added to 2.5ml of the diphenylamine reagent and the mixture was heated for 5 minutes in a boiling water bath. The sample was cooled and read at 540nm on a spectrophotometer (Varian Cary 219). The DNA concentration was calculated by comparing the value with a standard curve of salmon sperm DNA solution.

Restriction of total DNA and chloroplast DNA

Total DNA (10µg) and chloroplast DNA (2µg) were restricted overnight with *Hind III, Eco RI, Msp I, Hpa II* and *Pst I* [GIBCO, BRL (10 units/µg for total genomic DNA and 5units/µg for chloroplastic DNA)] using the restriction buffer (10X) provided by the company at 37^0C (Maniatis *et al*, 1990) to investigate site specific methylation of nuclear and chloroplastic DNA. Characteristic features of respective enzymes that were used are as follows: *Hind III* recognizes the sequence AAGCTT and does not cleave DNA when either the 5'A or the C residue is N^6-methyladenine or 5-methylcytosine, respectively. *Eco RI* recognizes the sequence GAATTC and does not cleave DNA when either the 3'A or the C residue is N^6-methyladenine or 5-methylcytosine, respectively. *Pst I* recognizes the sequence CTGCAG and does

not cleave DNA when either the A or the 5'C residue is N^6-methyladenine or 5-methylcytosine, respectively. *Msp I* recognizes the sequence CCGG and does not cleave DNA when the 5' C residue is 5-methylcytosine, but cleaves DNA when the 3'C residue is 5'-methylcytosine. *Hpa II* (isoschizomer of *Msp I*) recognizes the sequence CCGG and does not cleave DNA when the 3'C residue is 5-methylcytosine or either C residue is 4-methylcytosine.

Southern blotting

Southern blotting was done according to the manufacturer's (Amersham) protocol. 10µg restricted genomic DNA and 2µg restricted chloroplastic DNA in sample buffer (0.25% xylene cyanol and 0.25% (w/v) bromophenol blue) were electrophoresed on an 0.8% agarose TAE gel in 1X TAE buffer at 100V(constant voltage) for 3hr. After electrophoresis, the agarose gel was placed in 0.25M HCl until the front dye changed color from blue to yellow, and was then left for an additional 10 minutes. The gel was rinsed in distilled water and placed in denaturation buffer [1.5M NaCl and 0.5M NaOH] for 30 minutes at room temperature with shaking, then the gel was rinsed in distilled water and placed in neutralization buffer [1.5M NaCl, 0.5M Tris-HCl pH 7.2 and 0.001M EDTA] for 30 minutes at room temperature with shaking. The gel was then transferred in 20X SSC [3M NaCl and 0.3M sodium citrate] with the aid of a capillary set up (Maniatis *et al*, 1990) to Hybond N membrane. After the transfer, the membrane was briefly washed in 2X SSC and baked at 80^0C for 2hr under vacuum to cross link the DNA.

Northern blotting

Since the majority (90%) of total RNA comprises rRNA, it is difficult to study changes in rRNA expression on the basis of equal loading of total RNA. Therefore, in this study, the RNA/DNA ratio was first calculated and the total RNA was loaded on the basis of equal amounts of DNA (5µg). The RNA samples were heated at 65^0C for 5 minutes in the following solution: RNA (final volume 6-8 µl), formamide (deionized) 12.5 µl, 10X MOPS buffer 2.5µl, 4µl formaldehyde (37%) and chilled on ice. Then 2.5µl 50% (v/v) glycerol, containing 0.1 mg/ml bromophenol blue was added. RNA was electrophoresed on a 1.5 % agarose formaldehyde gel [1X MOPS buffer and 37% (v/v) formaldehyde], at 70V (constant voltage) in 1X MOPS running buffer [.02M MOPS, 5mM NaoAc and 1mM EDTA]. Total RNA from the gel

was transferred on to the Hybond C membrane with aid of capillary transfer set up (Maniatis *et al*, 1990). After the transfer was complete the membrane was briefly washed in 2XSSC and baked at 80^0C for 2 hr under vacuum to cross link the RNA (Fourney *et al*, 1988).

Amplification of nuclear and chloroplastic rDNA inserts

Inserts from *Eco RI* cloning site of pUC 11-5, (partial sequence of the clone showed 99% homology with tobacco's 23S rRNA and intergenic spacer between the 23S and 4.5S rRNA, Johnson-Flanagan, unpublished) and inserts from the *Pst I* cloning site of pMF2 clone, containing 26S, 17S and 5.8S rRNA of *Neurospora crassa*,(Free *et al*, 1979) were amplified by the PCR. The PCR condition were as follows: 10 µl Taq amplification buffer (10X), 2µl primer (Forward; 10pmol/µl), 2µl primer (Backward; 10 pmol/µl), 74µl H_2O, 0.5µl Taq Polymerase (5units/µl), 10µl dNTPs (.2mM) and 1µl DNA (1µg) were used for the PCR reaction. Different forward and backward primers were used with respect to the unique cloning sites. For pMF2 *Pst I* primers and for Puc 11-5 *EcoR I* primers were used.

Probe preparation

500 ng of amplified inserts were labelled with α-^{32}P-dCTP using the random priming kit (Amersham) which is based on random-primer method of (Feinberg and Vogelstein, 1983). The probes used for hybridization had specific activity of 1.5-1.7X 10^6 cpm/ml.

DNA/RNA blot hybridization

Hybridization was done according to the manufacturer's (Amersham) protocol. The blot was immersed in prehybridization buffer: 5X SSPE (made from 20X SSPE) [20X SSPE: 3.6M NaCl, 0.2M sodium phosphate, 0.02M EDTA pH7.7], 5X Denhardt's solution [100X: 2%(w/v) BSA, 2%(w/v) Ficoll, 2% (w/v) PVP], 0.5 % (w/v) SDS and 0.5 ml of a 1mg/ml solution of sonicated salmon sperm DNA (that had been denatured by heating at 100^0C for 5 minutes and chilled on ice). The membrane was prehybridized at 42^0C for 1 hour. Following prehybridization, the denatured ^{32}P probe (denatured by heating at 100^0C for 5 minutes and chilled on ice) was added to the prehybridization solution and incubated for 12-18 hr at 42^0C. Following hybridization, the filter

was washed in 2X SSPE and 0.1%(w/v) SDS at room temperature for 10 minutes and then another 15-20 minutes in 0.1% SSPE and 0.1% SDS at room temperature. The filter was removed and wrapped in SaranWrap and autoradiographed at -80^{0}C.

RESULTS

Nucleic acid content

The amount of total DNA per gram of fresh weight in mature leaves did not change with cold acclimation (Fig 3a). However, there was slight increase in the amount of total DNA per gram of fresh weight in acclimated young leaves as compared to non-acclimated young leaves. The amount of total RNA per gram of fresh weight increased in both mature and young leaves with acclimation when compared with non-acclimated leaves. This increase in RNA was greater in young leaves than in mature leaves.

Northern analysis

Figure 3b shows the bands that hybridized to the nuclear rDNA probe and the chloroplastic rDNA probe. Northern blots loaded on the basis of equal DNA content, showed that both nuclear and chloroplastic rRNAs were in high abundance in acclimated mature and young leaves as compared to non-acclimated leaves (Fig. 3b). This increase of both chloroplastic and nuclear rRNA abundance was higher in young leaves than in mature leaves.

Southern analysis of total DNA probed with nuclear rDNA

Southern blots of total DNA restricted with *Hind III* or *Eco RI* (Fig. 3c) showed similar hybridization patterns of nuclear rDNA in acclimated and non-acclimated mature and young leaves. Similarily, *Pst I* (Fig. 3d), *Msp I* and *Hpa II* (Fig. 3e) all failed to produce different restriction fragments between the various leaves. Comparison made between the isoschizomers, *Msp I* and *Hpa II*, indicated the presence of internal cytosine methylation at CCGG sequences of nuclear rDNA, as *Hpa II* restriction fragments were larger relative to the *Msp I* fragments (Fig. 3e).

Southern analysis of chloroplast DNA probed with chloroplast rDNA

Hybridization signals of chloroplastic rDNA (Fig 3f) restricted with *Hind III* or *Eco RI* did not reveal any differences between acclimated and non-acclimated mature and young leaves. However, hypomethylation at *Pst I* sites were seen with acclimation in both mature and young leaves. In addition, comparison of hybridization signals between non-acclimated mature and young leaves, showed young leaves having many *Pst I* sites as compared to mature leaves again indicating hypomethylation. The isoschizomeric enzymes, *Msp I* and *Hpa II* did not reveal any methylation differences in mature and young leaves with acclimation (Fig 3g). However, hybridization signals between *Msp I* and *Hpa II* restriction were different, with hybridization signals at higher molecular weight fragments in *Hpa II* restrictions relative to those in *Msp I* restrictions indicating that the internal cytosine residues were methylated.

DISCUSSION

Increased RNA expression (Paldi and Devay, 1977; Sarhan and Chevrier, 1985) and protein synthesis are two of the most important physiological changes that occurs in acclimated plant cells developing cold tolerance. Zvereva and Turnova (1985) have shown that when rRNA synthesis is inhibited, freezing tolerance is reduced from -18^0C to -13^0C in acclimated plants. Increases in rRNA and sRNA (mRNA and tRNA) have been reported to accompany acclimation (Sarhan and Chevrier, 1985; Laroche *et al*, 1992). This has been further supported by the fact that RNA polymerase I is more active than RNA polymerase II during acclimation in winter wheat (Sarhan and Chevrier, 1986). Similarily, results from the present study show increases in RNA during acclimation.

Increases in DNA content have also been shown in winter wheat (Teraoka, 1973), apple twigs (Li and Weiser, 1969) and in suspension cells of *Brassica napus* during the induction of freezing tolerance (Johnson-Flanagan and Singh 1987, 1988). However, in the present study there was no significant change in total DNA content in mature leaves with acclimation when compared to the control. In acclimated young leaves, there was a slight non-

significant increase in total DNA content when compared to the control (Fig 3a).

Studies of rRNA changes during cold acclimation have been restricted to nuclear encoded or total rRNA (Paldi and Devay, 1977; Sarhan and Chevrier, 1985). The present study shows that abundance of both nuclear and chloroplastic rRNA was high during cold acclimation.

Both nuclear (Scott *et al*, 1984; Flavell *et al*, 1986; Watson *et al* 1987) and chloroplastic (Ngernparsirtsiri et al, 1988a,b) genes have been shown to be methylated in higher plants. In the present study, differences between *Msp I* and *Hpa II* restriction digests were noted in nuclear and chloroplastic rDNAs. These two enzymes recognize the same sequence but their restriction pattern differs if there are methylation differences. Therefore, the hybridization signals that were observed in *Hpa II* restriction indicated that internal cytosine methylation were present at CCGG sequences in both rDNAs. This internal cytosine methylation did not change with acclimation in either mature or young leaves.

Alteration of methylation of specific sequences is important during plant development (Watson *et al*, 1987). Bird *et al*, (1981) reported hypomethylation of rDNA during *Xenopus laevis* development. Laroche *et al*, (1992) have shown that low temperature induced differential methylation of nuclear ribosomal genes occurs at *Eco RI* sites in *Brassica napus*. They suggested that this site specific methylation of *Eco RI* sites in rDNA might be related to the vernalization process and not to the development of freezing tolerance. In the present study, similar site specific methylation at *Eco RI* sites was not observed. This probably was a result of different acclimating temperature (2^0C), developmental stage (2 week old plant material), light conditions (350µmol m^{-2} s^{-1}) and acclimation time (28 weeks). Also, they did not mention which stage of leaves were used in their study.

Chloroplast genes have been shown to undergo changes in methylation during chloroplast biogenesis. For example, in tomato, the transition from chloroplast to chromoplast showed that low transcriptional activity of *lsu* and *psbA* correlated with hypermethylation (Ngernprasirtsiri *et al*, 1988a ; Kobayaschi *et al*, 1990). In non-photosynthetic amyloplasts, hypomethylation of 16S rRNA and *psbA* genes correlated with their active

transcription (Ngernprasirtsiri *et al*, 1988b). In the present study, differences in methylation of *Pst I* sites was observed in chloroplast rDNA. Acclimation was associated with hypomethylation in both mature and young leaves. In addition, the young leaves had less methylation at the *Pst I* sites as compared with the mature leaves. This suggests that hypomethylation of chloroplastic *Pst I* sites is associated with both growth and acclimation.

In conclusion, this is the first study that shows that chloroplastic and nuclear rRNA increase in a co-ordinated manner during low temperature acclimation with higher transcript abundance in young leaves in comparison to mature leaves. Although methylation was detected in both nuclear and chloroplastic rDNA, only hypomethylation of the Pst I sites of chloroplastic rDNA could be correlated with the increase in rRNA associated with acclimation.

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Figure 3a Total nucleic acid content in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Values are the means from 3 different experiments ± SD.

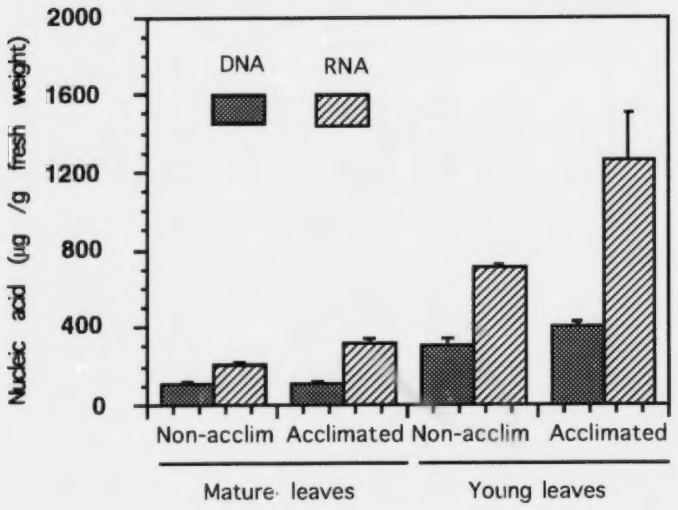

Nucleic acid (µg /g fresh weight)
2000
1600
1200
800
400
0
DNA
RNA
Non-acclim
Acclimated
Non-acclim
Acclimated
Mature leaves
Young leaves

Figure 3b (left) Northern blot (probed with pMF2) showing the abundance of nuclear rRNA transcripts in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. A-non-acclimated mature leaves, B- non-acclimated young leaves, C-acclimated mature leaves and D-acclimated young leaves.

Figure 3b (right) Northern blot (probed with pUC 11-5) showing the abundance of chloroplastic rRNA transcripts in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. A-non-acclimated mature leaves, B- non-acclimated young leaves, C-acclimated mature leaves and D-acclimated young leaves.

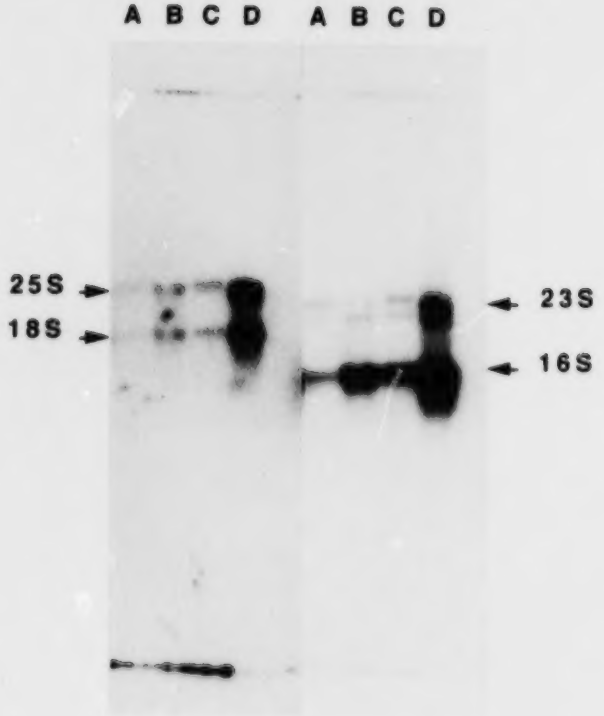
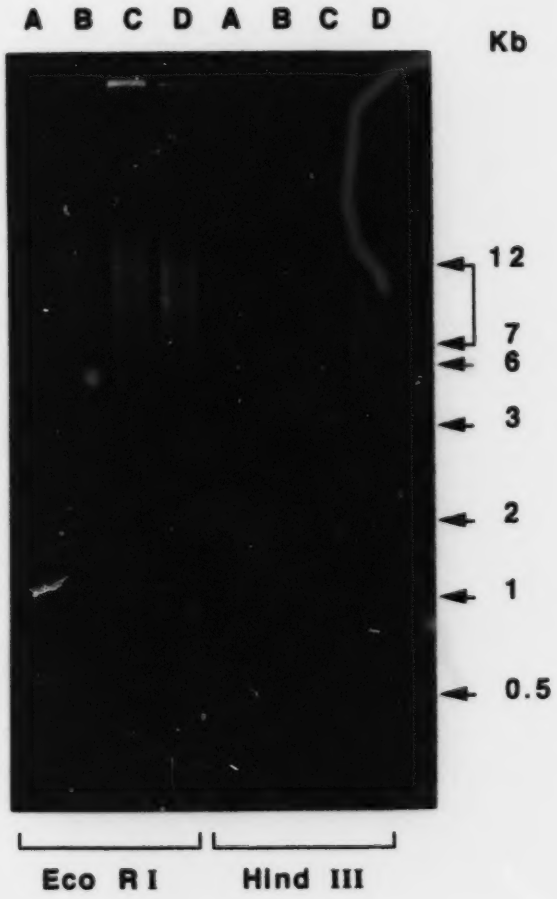

A B C D A B C D
25S
18S
23S
16S

Figure 3c Southern analysis of nuclear rDNA (probed with pMF2) in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Total genomic DNA was restricted with *Eco RI* and *Hind III,* (left) and hybridized with nuclear rDNA probe (right). A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.


A B C D A B C D
Kb
12
7
6
3
2
1
0.5
Eco R I
Hind III

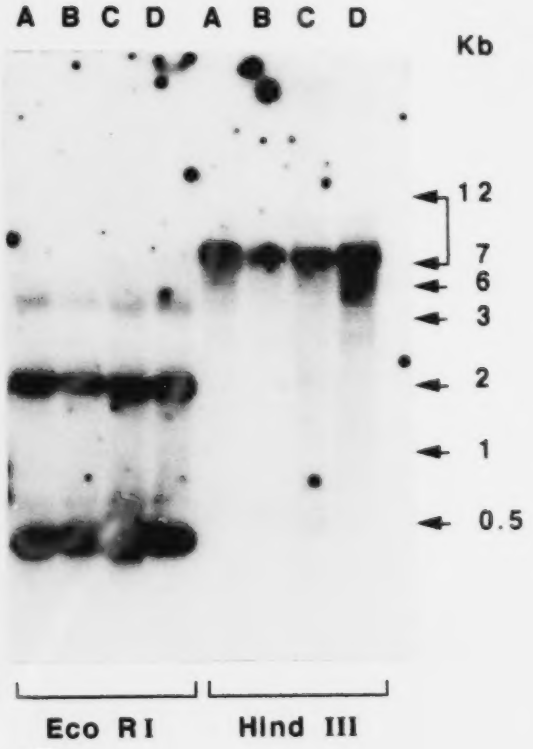
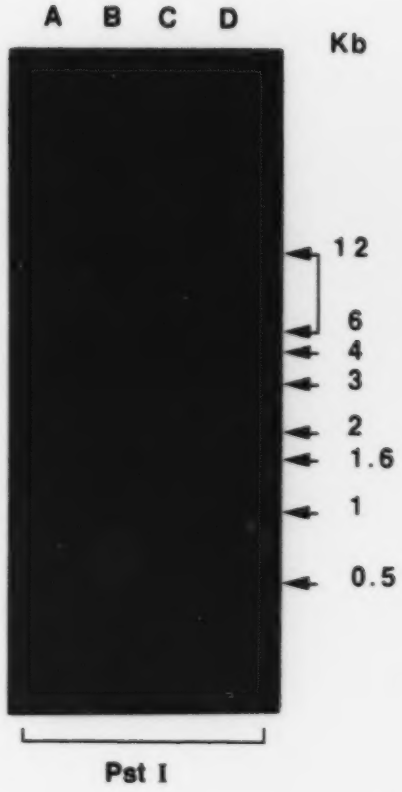

A B C D A B C D
Kb
12
7
6
3
2
1
0.5
Eco R I
Hind III

Figure 3d Southern analysis of nuclear rDNA (probed with pMF2) in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Total genomic DNA was restricted with *Pst I* (left) and hybridized with nuclear rDNA probe (right). A-non-acclimated mature leaves, B- acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.


A B C D
Kb
12
6
4
3
2
1.6
1
0.5
Pst I

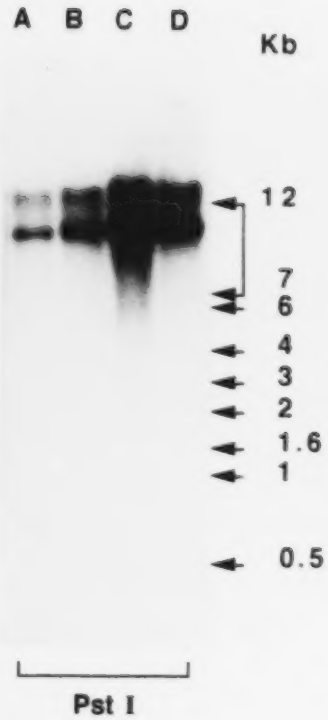

A B C D
Kb
12
7
6
4
3
2
1.6
1
0.5
Pst I

Figure 3e Southern analysis of nuclear rDNA (probed with pMF2) in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Restriction digests of *Msp I* and *Hpa II* on total genomic DNA (left) and the hybridization signals of nuclear rDNA (right). A-non-acclimated mature leaves, B- acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

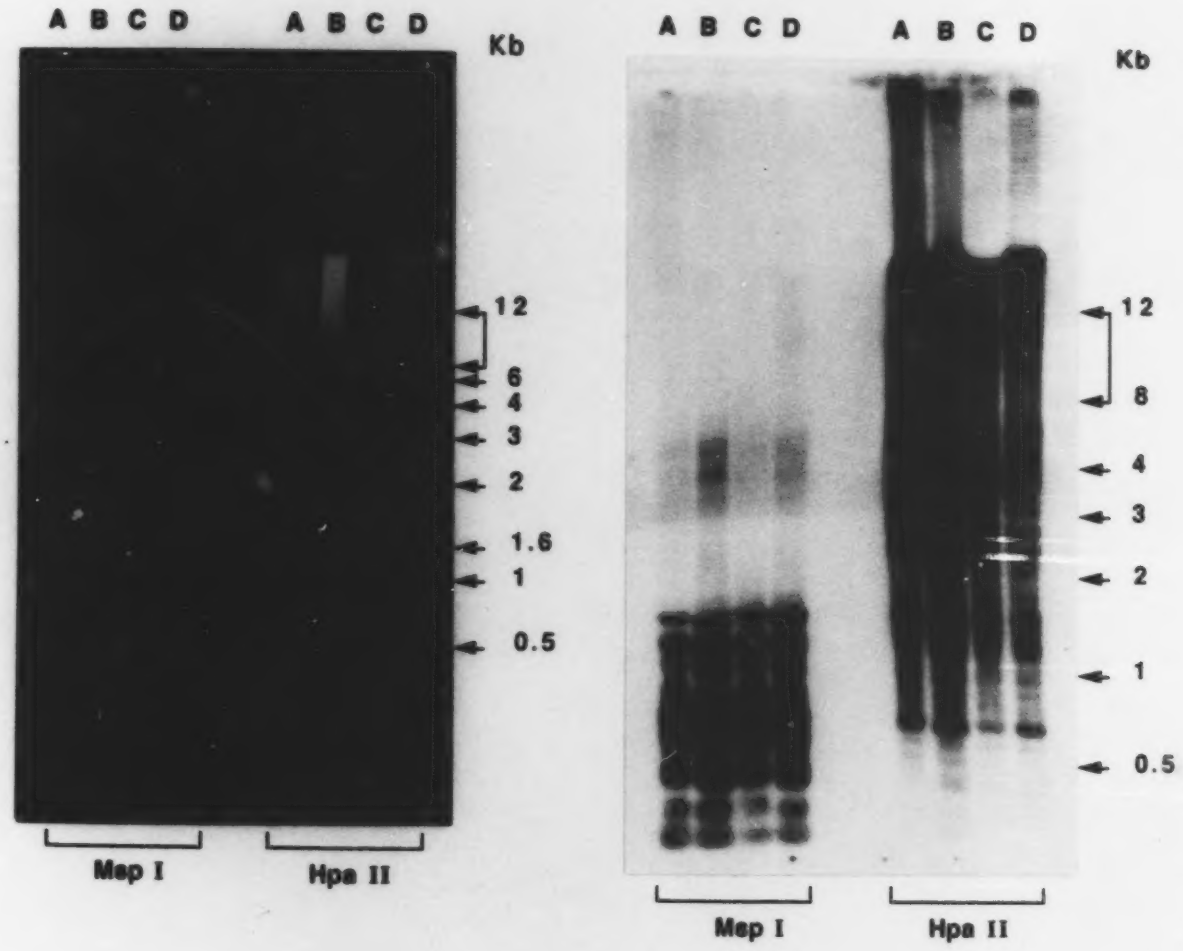

A B C D
A B C D
Kb
12
6
4
3
2
1.6
1
0.5
Msp I
Hpa II
A B C D
A B C D
Kb
12
6
4
3
2
1
0.5
Msp I
Hpa II

Figure 3f Southern analysis of chloroplast rDNA (probed with pUC 11-5) in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Restriction digestions of *Hind III,, Eco RI* and *Pst I* on chloroplstic DNA (left) and the hybridization signals of chloroplastic rDNA (right). A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

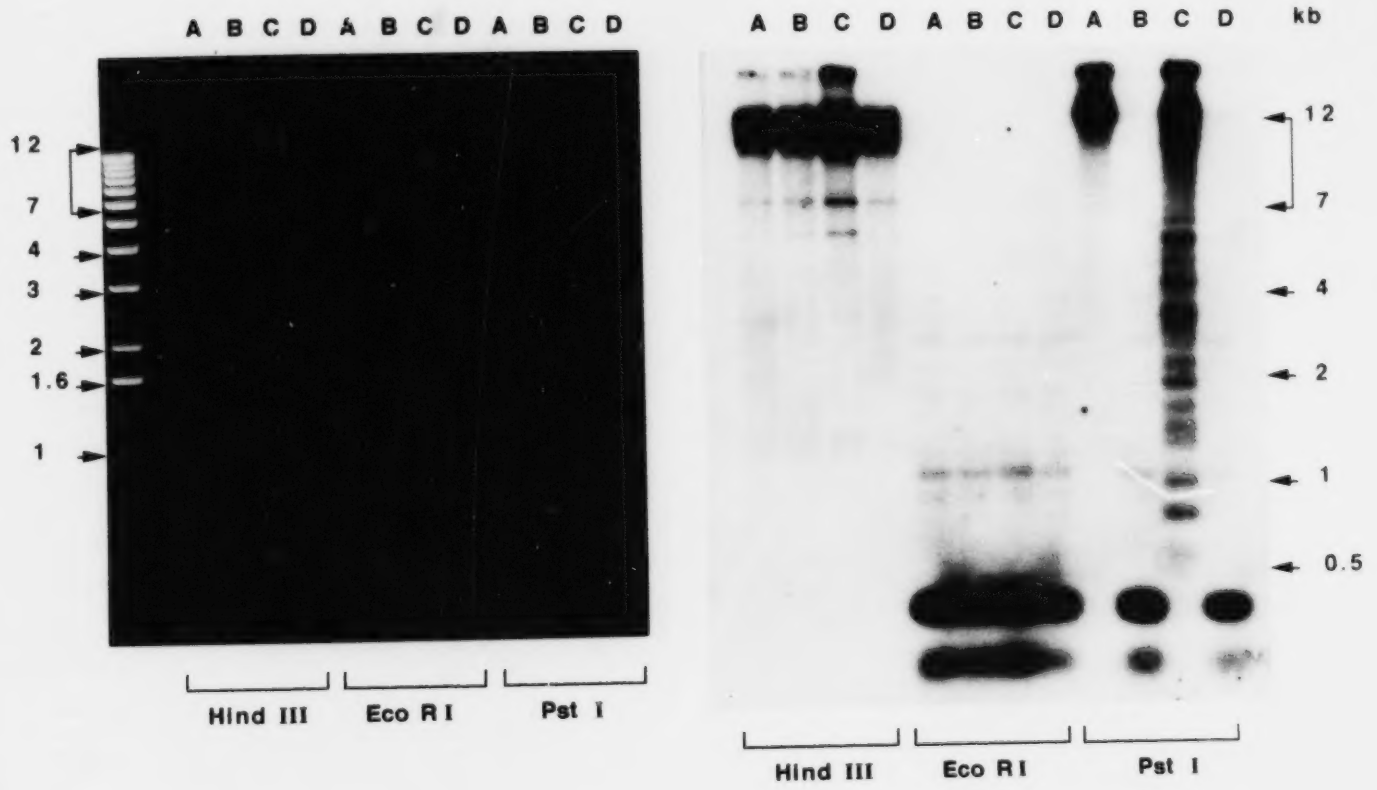
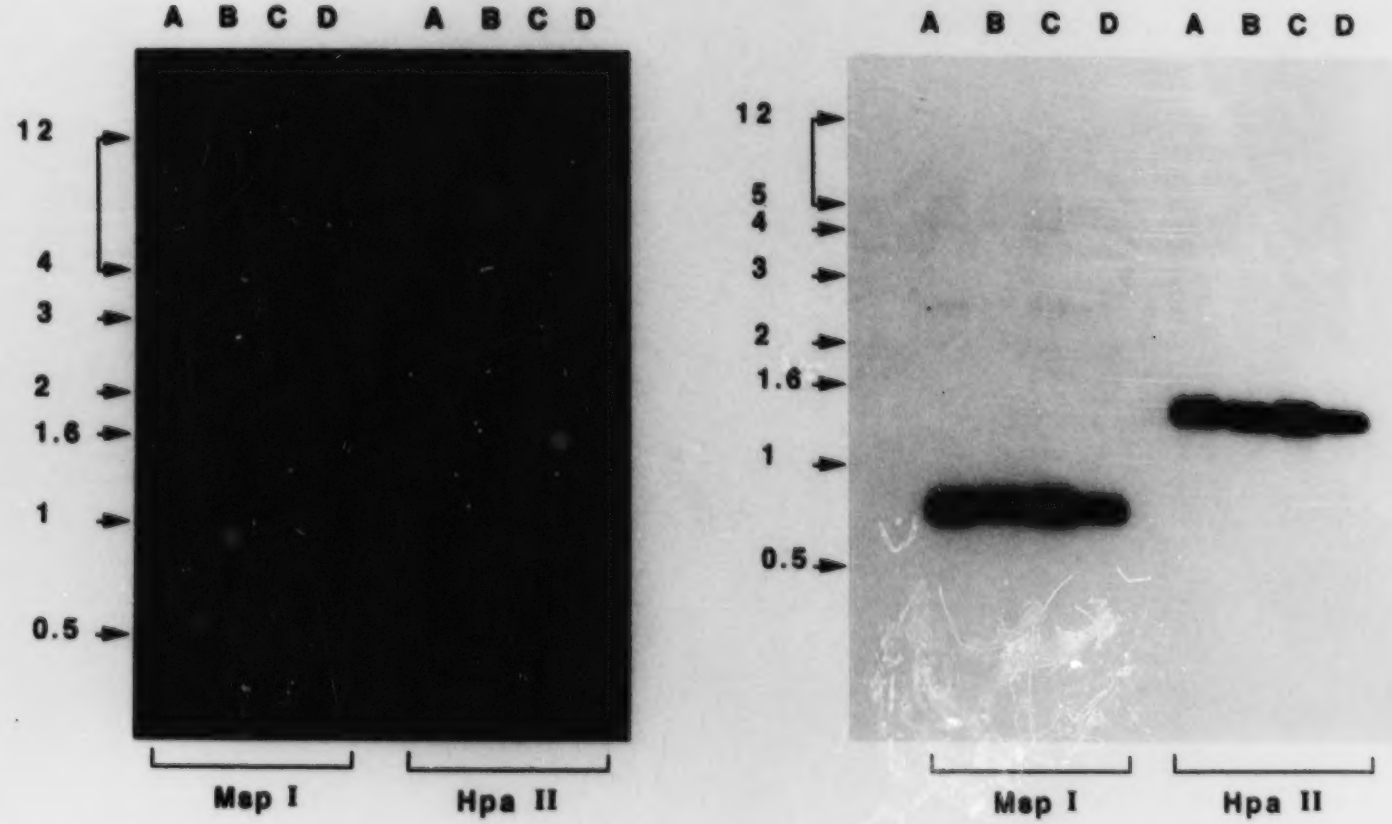

A B C D A B C D A B C D
12
7
4
3
2
1.6
1
Hind III
Eco R I
Pst I
A B C D A B C D A B C D
kb
12
7
4
2
1
0.5
Hind III
Eco R I
Pst I

Figure 3g Southern analysis of chloroplastic rDNA (probed with pUC 11-5) in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Restriction digestions of *Msp I* and *Hpa II* (left) and the hybridization signals of chloroplastic rDNA (right). A-non-acclimated mature leaves, B- acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.


A B C D A B C D
12
4
3
2
1.6
1
0.5
Msp I
Hpa II
A B C D A B C D
12
5
4
3
2
1.6
1
0.5
Msp I
Hpa II

CHAPTER 4

Low temperature acclimation results in differential expression of nuclear and chloroplastic genes encoding chloroplast proteins

INTRODUCTION

Many plant species acquire freezing tolerance with exposure to low non-freezing temperatures. Freezing tolerance involves changes in gene expression, including synthesis of new RNAs and polypeptides (Guy, 1990). Many cDNA clones from cold induced RNAs have been isolated and characterized in several plants including wheat (Houde *et al*, 1992), *Arabidopsis* (Gilmour *et al*, 1992; Kurkela and Frank, 1990), barley (Dunn *et al*, 1991; Cattivelli and Bartel, 1990), alfalfa (Wolfraim *et al*, 1993; Mohapatra *et al*, 1989) and *Brassica* (Weretilnyk *et al*, 1993). In wheat and alfalfa cultivars, expression of the cold induced mRNAs is directly related to the degree of freezing tolerance (Houde *et al*, 1992; Mohapatra *et al*, 1989) and the cold induced proteins are thought to be involved in acclimation of plants to low temperatures. While there has been a great deal of emphasis on the changes in gene expression during low temperature acclimation *vis* a *vis* novel genes, there has been little work on photosynthetic genes.

Low temperatures have been shown to affect photosynthesis but there are discrepancies in the published results. Photosynthetic capacity during low temperature acclimation can increase (Steffen and Palta 1987; Hurry *et al*, 1992; Oquist *et al*, 1993; Graham and Patterson, 1982; Berry and Bjorkman, 1980) or decrease (Somersalo and Krause, 1989; Maciejewska *et al*, 1987). Photosynthetic responses to low temperatures are species and cultivar dependent (reviewed by Huner *et al*, 1993). In addition, contrasting observations result from differences in the physiological age of the leaves that are compared. Aging during the low temperature acclimation accounts for most of the observed reduction in the rate of photosynthesis (Krol *et al*, 1984;

Boese and Huner, 1990). Both alteration of metabolism and changes in gene expression play a role in the photosynthetic response to low temperature.

Expression of photosynthesis related proteins requires the co-ordinate expression of nuclear and chloroplast encoded genes (Taylor, 1989; Mayfield, 1990; Susek and Chory, 1992). Plants are able to co-ordinate the accumulation of the chloroplast and nuclear polypeptides through a complex hierarchy of controls, including control of transcriptional rates and mRNA accumulation (Gruissiem, 1993). Much of the transcriptional control is mediated by phytochrome (Gruissiem, 1993; Gilmartin *et al*, 1990; Sexton *et al*, 1990; Mullet, 1988), which is a significant factor in the expression of the nuclear encoded photosynthetic genes. In addition to transcriptional control, the chloroplast encoded genes are regulated by mRNA stability and splicing (Gruissem, 1993; Gruissem *et al*, 1988; Link, 1988; Mullet, 1988). The expression of genes encoding components of the photosynthetic apparatus are coupled to morphological development and chloroplast biogenesis (Ellis, 1981; Taylor, 1989). Some of the regulatory genes respond to the developing chloroplast by fine-tuning structural gene expression according to the plant's photosynthetic need (Mayfield, 1990).

Ribulose 1,5 biphosphate carboxylase/oxygenase (RUBISCO) is an abundant soluble enzyme found in the stroma of chloroplasts. It has a multimeric structure composed of two types of subunits. The larger subunit (LSU), is encoded by chloroplast DNA and synthesized within the organelle. The smaller subunit (SSU), encoded by nuclear DNA, is synthesized in the cytoplasm as a higher molecular weight precursor polypeptide. This precursor is transported into the chloroplast, processed to its mature form and then combines with LSU to form the functional enzyme. Co-ordinate translation and post translational control further ensure stoichiometric accumulation of the chloroplast (LSU) and nuclear (SSU) encoded subunits of RUBISCO, respectively (Rodermel *et al*, 1988).

During cold acclimation, formation of two distinct forms of each of the LSU and SSU subunits occurs in cabbage (Shomer and Waisel, 1975). In rye, the structural and kinetic properties of RUBISCO have been shown to be altered, leading to an increased stability at low temperature (Huner and Macdonald, 1979). Low temperature stress has also been shown to partially

disrupt the co-ordination of *lsu* and *ssu* gene expression; *ssu* was more suppressed relative to *lsu* (Hahn and Walbot, 1989). Similarily, low temperature treatment results in suppressed SSU synthesis in etiolated *Brassica napus* seedlings (Meza-Basso *et al*, 1986) and suppression of both SSU and LSU, with SSU being more sensitive, in nontolerant rice (Shakya and Agrawal, 1993). In contrast, RUBISCO is induced in cold tolerant rice. However there is still apparent loss of co-ordination as proportionately more SSU accumulated (Shakya and Agrawal, 1993).

The most abundant chlorophyll-binding polypeptide is the light harvesting CAB (chlorophyll a/b protein) (Bennett, 1981). The protein organizes about one-third of the chlorophyll a and possibly all of the chlorophyll b molecules of the membrane into complexes that absorb light and transfer the resulting excitation energy to the two photosystems. Interestingly, 5% or less of *cab* message is sufficient to allow normal protein accumulation binding of chl a and b and support normal photosynthesis (Flachmann and Kuhlbrandt, 1995). In pea, turnover of CAB was shown to be light dependent and the half life of the protein when plants were shifted from light to dark was 90 minutes (Bennett, 1981). Low temperature acclimation in potato leaves have been shown to reduce the number of chlorophyll molecules per LHC polypeptide (Steffen and Palta, 1987), thereby reducing antenna size. In addition, changes in the structure of LHC protein complexes have been shown, where a shift from oligomeric to monomeric forms have been observed in response to low temperature in monocot species (Huner *et al*, 1987).

One of the most actively synthesized membrane bound proteins is the *psbA* gene product, known as the D1 protein or Q_B-binding protein (Edelman and Reisfeld 1978; Mattoo *et al* 1984). D1 protein exhibits a high rate of light dependent turnover in plants with half life of 2.5hr (Aro *et al*, 1993). *psbA* gene expression has been shown to be unaffected by low temperature treatment (Hahn and Walbot, 1989).

The objective of this portion of the thesis was to examine the effect of low temperature acclimation and not low temperature stress (as other studies have done) on expression of nuclear and chloroplastic genes encoding chloroplast proteins. The expression of both chloroplastic and nuclear

ribosomal RNAs have been shown to increase during low temperature acclimation in both mature and young leaves of *Brassica napus* cv. Jet Neuf (Chapter 3). In this report, differential expression of *lsu, ssu, cab* and *psbA* genes in the two leaf ages was examined during three weeks of low temperature acclimation. Two nuclear encoded (*ssu* and *cab)* and two chloroplast encoded (*lsu* and *psbA*) genes were studied at the mRNA level and their pattern of accumulation investigated at the protein level in mature and young leaves.

MATERIALS AND METHODS

Growth condition

Growth conditions and harvesting of plant material were same as in Chapter 3. Since plants grow more slowly at lower temperatures, both acclimated and non acclimated plants reached the same developmental stage at different chronological ages. The non-acclimated plants reached the same developmental stage as the acclimated plants after 23-24 days. Mature and young leaves from non-acclimated plants were harvested at 23-24 days, and this represented the time 0, control. The acclimated mature and young leaves were harvested at 42-43 days. These were decided on the basis of their leaf lengths (Chapter 2) in their four leaf stage of development.

Chlorophyll extraction

Chlorophyll was extracted in cold 80% (v/v) acetone from leaf material that had been freeze dried in the dark (Arnon, 1949). Leaves (100mg) were ground in 100µl of 80% (v/v) cold acetone. This step was repeated until no green color remained in the tissue. The concentration of chlorophyll and chlorophyll a/b ratios were determined by spectrophotometry (Varian Cary 219) at 663nm and 645nm for chlorophyll a and b, respectively.

Protein determination

One gram of leaves were solubilized in 500µl Laemmli SDS extraction buffer (Laemmli, 1970) and heated in a boiling water bath for 5 minutes. The sample was centrifuged at 13,000 rpm for 10 minutes on a bench microcentrifuge. 50 µl of the supernatant was precipitated with 10% TCA and

dissolved in 0.1 NaOH. The protein concentration was measured according to Bradford (1976).

^{35}S-methionine incorporation

The wounding method of Cooper and Ort (1988) was used. The upper surface of leaves were abraded with sand paper and 10µCi of ^{35}S methionine (in 10 µl 0.01% Tween 20) was evenly applied by spreading with a cotton-wool swab. Mature and young leaves on intact acclimated (4^0C) and non-acclimated plants (20^0C) were labeled for 1, 2, 3, and 4hrs, the leaves were harvested, extensively washed in distilled H_2O, frozen in liquid nitrogen and stored at -80^0C. Radio-labeled proteins from mature and young leaves were extracted in Laemmli extraction buffer (Laemmli, 1970) supplemented with 0.2 mM PMSF, and kept on ice. The crude extract was boiled for 5 minutes and centrifuged. The supernatant was used for SDS-PAGE and TCA precipitation. The protein concentration was determined as above. The incorporation of ^{35}S methionine into TCA precipitable protein was determined by spotting 5µg of the protein sample on 3MM paper discs (Whatman) followed by precipitating the protein with 10% TCA for 30 minutes followed by 5% TCA for 15 minutes, rinsing in 95% ethanol, drying and counting in a scintillation counter (Isocap 300, Searle Analytic Inc.).

RNA extraction and Northern analysis

Extraction of total RNA and Northern analysis were the same as in Chapter 3. The gels were stained with ethidium bromide prior to transfer and the blots were probed with nuclear rDNA to ensure equal loading (Fig. 4a)

cDNA Probes

The cDNA fragments from clones (Appendix-1) were gel purified and electroeluted, after restriction digestion of the plasmids (Maniatis *et al*, 1990). Probes were radiolabeled with α-^{32}P-dCTP by random priming (Amersham) as in Chapter 3.

Hybridization and quantification

Hybridization was carried out at 42^0C as in Chapter 3. Autoradiographic exposure was carried out at -80^0C, with intensifying screens on Kodak X-ray

film. The relative abundance of the transcripts from various genes were quantified from the autoradiographs. Volume analysis of the hybridized bands were done on a GS-670 Imaging densitometer (Biorad Image Analysis System). The values were plotted on the graph. For each transcript, the maximum value was normalized at 100 and the other samples normalized accordingly.

Western blotting

Equal amounts of total SDS soluble protein (20µg) or total SDS soluble proteins on equal chlorophyll content (5 µg) were separated by SDS-PAGE (7-12% gradient gel) at 120V (constant voltage) for 8hr in Laemmli's running buffer (Laemmli, 1970) using a BioRad electrophoresis unit. After electrophoresis, proteins were electroblotted to a nitrocellulose membrane (Hybond C, Amersham) at 70V for 2 hr in transfer buffer [10mM $NaHCO_3$, 3mM Na_2CO_3 in 20% methanol, pH 9.8] (Dunn, 1986). Blots were stained with Ponceau S (Fig. 4b) to ensure complete transfer. The the blots were reacted with polyclonal antibodies to either PSBA, CAB, LSU or SSU (at 1:500 or 1:1000 dilution Appendix-1). Protein-antibody complexes were visualized by incubation with alkaline phosphatase-linked, goat anti-rabbit-IgG (Sigma, 1:5000 dilution), followed by reaction with 5 bromo-4-chloro-3 indolyl phosphate toluidine salt and p-nitroblue tetrazolium chloride substrate. The relative abundance of the polypeptides for various proteins were quantified from the immunoblots using a GS-670 Imaging densitometer (Biorad Image Analysis System). For each polypeptide the maximum value was normalized at 100 and the other samples normalized accordingly.

Protein biosynthesis and turnover

Labeling with ^{35}S-methionine was as above. To prevent from drying 10µl of distilled water was pipetted on to the labeling site every 15 minutes during the one hour labeling period. Four plants were labeled for each treatment and chased with cold methionine (15µl of 1mM non-radioactive methionine in 0.01% Tween 20) at 0, 2, 4, 6, 8, and 10hr.

Radio-labeled proteins were loaded on the basis of equal radioactive counts (100,000 c.p.m.) and separated on a 7%-12% SDS PAGE gradient gel at constant voltage of 120 V for 8 hr as above. At the end of the run, the gels

were stained with Coomassie blue (0.1% Coomassie blue in 40% methanol and 10% acetic acid) followed by destaining (40% methanol and 10% acetic acid). The stained gels were impregnated with En^{3}hance for 60 minutes with gentle agitation. After the impregnation, the gels were rinsed with distilled water and soaked in 10% PEG_{8000} for 1 hour with gentle agitation. The gels were then dried and visualized by fluorography. Densitometry scans were done on a GS-670 Imaging densitometer (Biorad Image Analysis System).

Immuno-radioactive bands of SSU and CAB

To further examine the fate of LSU, SSU and CAB during the pulse chase experiment, radiolabelled proteins (200,000 c.p.m.) were loaded on a 7-12% SDS PAGE gradient gel and electrophoresed at 100V for 8hr. After electrophoresis, Western blots were done as above. The immunoblots were reacted with the specific polyclonal antibodies for LSU, SSU and CAB and the immuno-radioactive bands were excised and counted in a scintillation counter (Isocap 300, Searle Analytic Inc.).

RESULTS

Chlorophyll content

Acclimation led to lower chlorophyll contents in both leaves compared to the non-acclimated leaves. There were no significant differences in chorophyll content between mature and young leaves. Chlorophyll a/b ratio did not change in either cold acclimated mature leaves or young leaves during cold acclimation as compared to non-acclimated mature and young leaves (Table 4.1).

Total SDS soluble protein

Total SDS soluble protein content increased with acclimation in both mature and young leaves with a greater increase in young leaves on a fresh weight basis (Table 4.2). In comparison, on a dry weight basis both mature and young leaves showed increases in SDS soluble protein to a similar extent during low temperature acclimation (Table 4.2).

^{35}S-methionine incorporation in TCA precipitatble proteins

Rate of incorporation of ^{35}S-methionine was low in acclimated mature leaves whereas in the young leaves rate of incorporation was more (Figure 4m and Table 4.3). This indicates that the rate of protein biosynthesis was low in mature leaves and high in young leaves during cold acclimation.

Expression of lsu

The relative abundance of *lsu* mRNA was greatest in non-acclimated mature leaves. Acclimation led to decreased *lsu* mRNA in mature leaves and a small increase in *lsu* mRNA in young leaves (Fig 4c). This small increase appears to be significant as at the protein accumulation level there was the same small difference in the acclimated young leaves. The corresponding Western blots (Fig. 4d) showed greatest accumulation of LSU polypeptide in mature leaves of non-acclimated plants and relatively high amounts in the other leaves. Also noted was a high amounts of LSU relative to the message in acclimated mature leaves. This indicated low turnover of LSU polypeptide or high turnover of *lsu* transcripts.

Expression of ssu

Low temperature acclimation was associated with low abundance of *ssu* mRNA in mature leaves and high abundance in young leaves with respect to non-acclimated leaves (Fig 4e). The corresponding Western blot showed (Fig 4f) a pattern of accumulation similar to that seen with the Northern blot. Comparison made between LSU and SSU indicate there was more LSU in both acclimated mature and young leaves. Ratio of LSU to SSU from the western blots were calculated. Taking the non-acclimated mature leaves ratio of LSU: SSU as 1:1, the ratio of LSU:SSU in acclimated mature leaves were 1.3:1, in non-acclimated young leaves 1.2:1 and acclimated young leaves 1.2:1. This indicates that LSU and SSU accumulated in proportion.

Expression of Cab

Cab mRNA decreased with acclimation in the mature leaves and increased in young leaves (Fig 4g). The corresponding Western blot (Fig 4h) loaded on the basis of equal protein showed that, relatively less CAB polypeptides accumulated in acclimated mature leaves. Although CAB

polypeptides did accumulate more in acclimated young leaves, it was not to the same extent as the relative abundance of *cab* mRNA in acclimated young leaves. CAB levels were higher in acclimated mature leaves and in non-acclimated young leaves relative to the message levels.

Western blots (total protein was loaded on the basis of equal chlorophyll content) showed that the total number of CAB polypeptides per µg of chlorophyll was high in acclimated mature leaves as compared to young leaves. This indicates decreased antenna size of LHCII (Fig. 4i).

Expression of psbA

Northern blots showed lower abundance of *psbA* mRNA in mature leaves and higher abundance in young leaves with low temperature acclimation relative to the non-acclimated leaves (Fig 4j). The corresponding Western blot (Fig 4k) showed lower accumulation of PSBA polypeptide in acclimated mature leaves and slightly higher accumulation in acclimated young leaves when compared to non-acclimated leaves.

Protein biosynthesis and turnover

Protein biosynthesis and turnover kinetics were studied for LSU, SSU and CAB polypeptides as there appeared to be disproportionately low levels of transcripts relative to the polypeptides in acclimated mature leaves. On the other hand, acclimated leaves had high levels of *ssu* relative to SSU. LSU, SSU and CAB failed to show any turnnover in mature and young leaves (An example of such an autoradiogram of young leaves is shown in Fig. 4l). As such, data over a 10hr chase period were pooled as a measure of biosynthesis Table 4.4, 4.5, 4.6 and 4.7). Although the results differed between the densitometry scan and the immunoreactive bands, the general trends were the same (Table 4.8). LSU synthesis was suppressed by 36-47% and CAB synthesis was suppressed 17-30% with acclimation in mature leaves. In contrast, SSU biosynthesis increased 15-25% in acclimated mature leaves. In the young leaves, SSU biosynthesis did not vary with acclimation.

DISCUSSION

The present study on photosynthetic gene expression during low temperature acclimation in *Brassica napus* cv Jet Neuf showed a number of

differences between the mature and young leaves during cold acclimation: Gene expression decreased in the mature leaves, whereas it generally increased in the young leaves; antenna size of LHCII decreased in the mature leaves and remained unchanged in the young leaves; *lsu* and *ssu* gene expression were coupled in mature leaves, but were not in young leaves; and polypeptide levels generally reflected gene expression in the young leaves and did not in the mature leaves.

lsu, ssu, cab and *psbA* were differentially expressed in the two leaf ages, with high expression levels in the non-acclimated mature leaves and low expression levels in the acclimated mature leaves. Although the greatest decrease in gene expression occurred in the mature leaves, this was not the result of senescence. Yellowing is the most visible characteristic of senesecence. Chlorophyll disappearance, a decrease in the chlorophyll a/b ratio and loss of RUBISCO are the principle criteria of senescence for most researchers (Gepstein, 1979; Back and Richmond, 1971; Jenkins and Woolhouse, 1981). During growth and development at cold hardening temperatures, the chlorophyll content on a dry weight basis goes down in winter rye (Huner *et al*, 1984) and chlorophyll a/b ratio in puma rye are unaltered(Krol and Huner, 1985) Similar results were obtained in the present study. Both leaf ages showed a decrease in chlorophyll content on a dry weight basis with acclimation and no change in the chlorophyll a/b ratio. Other results supporting a lack of senescence includes the increase in rRNA expression (Chapter3) and the increase in total protein in both leaf ages during acclimation. These changes and the fact that the leaves were matched for developmental stage clearly indicates that the changes in gene expression are in response to low temperature.

It is unclear why the mature leaves undergo such a dramatic decrease in photosynthetic gene expression with acclimation. Certainly, other genes continue to be expressed at a high rate, as rRNA levels increase with acclimation (Chapter 3), and protein levels increase. Both these changes have been reported to be associated with acclimation (Steffan and Palta, 1986; Sarhan and Chevrier, 1985; Palta and Devay, 1977).

Another difference noted between the young and mature leaves was the reduction of antenna size of LHCII. Reduced antennae size of LHCs been

reported to accompany acclimation in *Solanum commersoni* (Steffan and Palta, 1986).

Unlike the mature leaves, photosynthetic gene expression generally increased in response to acclimation in the young leaves. However, there appears to be a loss of co-ordination between *lsu* and *ssu* gene expression. From the western blot analysis of LSU and SSU, the calculated ratio showed that LSU and SSU accumulated in proportion suggesting no limitation on the capacity for carbon metabolism. In order to analyze the different effect on SSU and LSU synthesis during cold acclimation, the ratio of ^{35}S methionine incorporation in LSU and SSU synthesis can be determined. In this study the ratio of ^{35}S methionine incorporation (LSU: SSU) could not be calculated as immunoprecipitation of RUBISCO was not carried out.

Results from studies on cold stress suggest there is a loss of co-ordination at the protein level. My results show that this loss of co-ordination does not occur during cold acclimation. With respect to cold stress, relatively lower rates of *ssu* transcription and low rates of SSU synthesis have been reported in rice (Hahn and Walbot, 1989), and relatively lower rates of SSU synthesis have been measured in etiolated *Brassica* seedlings (Meza-Baso *et al*, 1986). Studies of low temperature acclimation show more SSU accumulating than LSU (Shakaya and Agarwal, 1993). Results from the present study show that *lsu* transcript levels were low relative to *ssu* but this had no effect on the LSU:SSU ratio during cold acclimation. Thus, post-transcription controls may have led to differences in protein accumulation.

Finally, whereas photosynthetic gene expression generally correlated quite well with protein accumulation, this was not the case for *ssu, lsu, psbA* and *cab* in the mature leaves and *ssu* in the young leaves. As the protein levels were higher than the message levels, polypeptide biosynthesis rates and turnover was examined. The results also show no turnover in a 10hr chase. Similarily, Hahn and Walbot (1989) failed to show turnover of LSU and SSU during a 48hr chase but no studies on turnover of PSB A and CAB were examined in their study. The results of the present study suggest that LSU and CAB levels in mature leaves could be maintained with low rates of biosynthesis. In light of this, SSU biosynthesis would be expected to be similarly reduced, but it was not. The fact that SSU biosynthesis increased

with acclimation in the mature leaves and remained the same in young leaves may reflect low accumulation of SSU relative to LSU in these leaves. Thus, SSU biosynthesis is required to equalize the relative amounts of SSU and LSU.

Acknowledgment:

cDNAs of *ssu* pSS65 (pea rbcs) (Coruzzi *et al* , 1983), *lsu* pJZA4 (Spinach rbcl) (Erion *et al* ,1981),*psbA* pMBB1.83 (mung bean psbA), (Palmer *et al* , 1982) and *cab* pAB56 (pea cab) (Cashmore, 1984), were all a kind gift from Dr Ken Ko., Queens University, Kingston, Ontario, Canada. Polyclonal antibodies were all a kind gift from Dr. Shawn Hemmingson for RUBISCO, from Dr. John Mullet for PSBA and from Dr Shimon Gepstein and Dr Dyson for LHCII antibodies. The author is very grateful to all of them.

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TABLE 4.1 Total chlorophyll content (mg/g dry wt) in mature and young leaves from three weeks acclimated and non-acclimated plants. A- non-acclimated mature leaves, B- acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves. Values are the means of 6 determinations $\pm$ SD.

Growth temperature	Leaf number	chl(mg/g dry.wt)	chl a/b ratio
20^0C	A	8.4 $\pm$ 0.6	2.7$\pm$0.2
4-5^0C	B	4.7 $\pm$ 0. 3	2.8 $\pm$0.4
20 ^{0}C	C	8.1$\pm$ 0.2	2.7 $\pm$0.3
4-5^0C	D	5.1$\pm$ 0.7	2.7 $\pm$ 0.5

TABLE 4.2 Total-SDS soluble protein content in mature and young leaves of three weeks acclimated and non-acclimated plants. A- non-acclimated mature leaves, B- acclimated mature leaves, C-non-acclimated young leaves and D- acclimated young leaves. Values are the means of 3 determinations ± SD.

Leaf ages	Total SDS soluble protein (mg/g dry weight)	Total SDS soluble protein(mg/g fresh weight)
A	50.8 ±2.3	4.2 ± 0.09
B	71.1 ±3.5	9.3 ± 0.16
C	53.2 ± 5.7	5.3 ±0.26
D	71.3 ± 3.2	13.6 ± 0.63

Table 4.3 Rate of incorporation of ^{35}S-methionine (mean of 2 replicates) into 5mg protein in mature and young leaves of *Brassica napus* during low temperature acclimation. Regression values was determined from each slope of 0-4hrs of labeling (Figure 4m). A-non-acclimated mature leaves, B-acclimated mature leaves, C-Non-acclimated young leaves and D-acclimated young leaves.

	A	B	C	D
Slope(incorporation/hr)	43000	20000	34000	37000
Significance (r)	.97	.99	.97	.99

Table 4.4 Densitometry scan was done on the autoradiogram for LSU, CAB and SSU using the GS-670 Imaging densitometer (Biorad Image Analysis system). Values represent the OD of the radioactive bands during the pulse chase of 10 hours in acclimated and non-acclimated mature leaves. Values are from 3 replicates of 1 experiment.

	LSU		CAB		SSU	
Hours	Non-acclimated	Acclimated	Non-acclimated	Acclimated	Non-acclimated	Acclimated
0	17.2	11.3	5.2	4.1	4.8	7.6
2	18.6	12	7.3	3.6	5.6	5.3
4	16.1	15.4	5.2	6.4	5.0	6.3
6	17.3	10.5	5.0	4.0	4.3	5.9
8	17	5.5	5.1	1.7	5.1	4.6
10	16	12	7.1	5.0	6.1	6.4

Table 4.5 Densitometry scan was done on the autoradiogram for LSU, CAB and SSU using the GS-670 Imaging densitometer (Biorad Image Analysis system). Values represent the OD of the radioactive bands during the pulse chase of 10 hours in acclimated and non-acclimated young leaves. Values are from 3 replicates of 1 experiment.

	LSU		CAB		SSU	
Hours	Non-acclimated	Acclimated	Non-acclimated	Acclimated	Non-acclimated	Acclimated
0	17.9	16.4	24.1	15.7	3.4	2.6
2	30.1	16.1	27.5	17.0	5.2	1.9
4	25.2	16.7	26.7	17.7	2.7	4.3
6	24.3	18.9	30.2	18.4	3.4	3.7
8	19.7	20.2	25.1	19.9	2.9	4.8
10	22.6	21.1	24.3	16.2	3.1	2.9

Table 4.6 Radioactive counts from the radio-immuno reactive bands of LSU, CAB and SSU during the 10hr pulse chase in mature leaves of *Brassica napus* during low temperature acclimation. Each lane was loaded on the basis of equal radioactivity pooled from 3 different plants of 1 experiment (200,000 c.p.m.).

	LSU		CAB		SSU	
Hours	Non-acclimated	Acclimated	Non-acclimated	Acclimated	Non-acclimated	Acclimated
0	14244	12029	5072	3190	484	2066
2	17361	6642	4242	4222	309	2886
4	13286	10853	4210	3632	446	1004
6	14317	10840	2874	1897	267	1176
8	20332	4672	4073	2261	485	1784
10	20030	8263	3525	4734	769	1925

Table 4.7 Radioactive counts from the radio-immuno reactive bands of LSU, CAB and SSU during the 10hr pulse chase in young leaves of *Brassica napus* during low temperature acclimation. Each lane was loaded on the basis of equal radioactivity pooled from 3 different plants of 1 experiment (200,000 c.p.m.).

	LSU		CAB		SSU	
Hours	Non-acclimated	Acclimated	Non-acclimated	Acclimated	Non-acclimated	Acclimated
0	16882	17679	4969	4343	1620	856
2	24784	12698	7283	3746	849	514
4	12959	19152	5251	4055	493	696
6	17658	14937	6946	4634	516	782
8	21149	17404	6358	4129	508	1106
10	24257	13243	6925	4118	797	837

Table 4.8 Comparison of biosynthesis of LSU, SSU and CAB in acclimated mature and young leaves with non-acclimated mature and young leaves. Values were obtained from the mean of 10hrs chase of acclimated leaves divided by mean of 10hrs chase in non-acclimated leaves of both densitometry scan values and immunoreactive values.

Mature Leaves :

	Densitometry scan	Immunoreactive band
LSU	36% less	47% less
CAB	30% less	17% less
SSU	15% more	25% more

Young leaves:

	Densitometry scan	Immunoreactive band
LSU	22% less	20% less
CAB	34% less	34% less
SSU	same	same

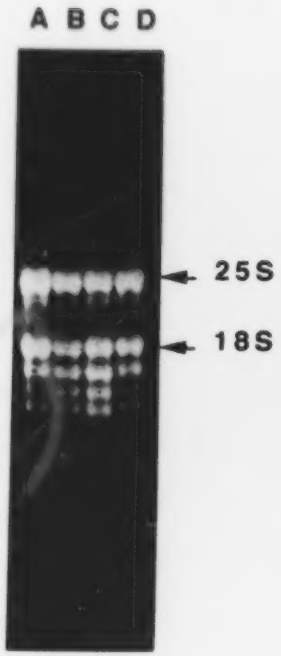
Figure 4a

Left

Total RNA (20µg/lane) separated on 1.5% agarose formaldehyde gel, stained with ethidium bromide prior to transfer to the membrane.

Right

An example of a Northern blot of total RNA (20µg/lane) probed with nuclear rDNA. A- non-acclimated mature leaves, B- acclimated mature leaves, C-non-acclimated young leaves and D- acclimated young leaves.


A B C D
25S
18S

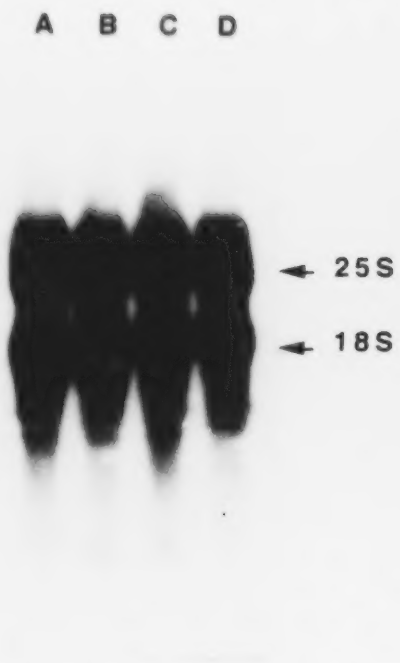

A B C D
25S
18S

Figure 4b Ponceau S stained blot of total SDS soluble protein, separated by SDS PAGE. A- non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves. M-Molecular rainbow markers (Amersham).

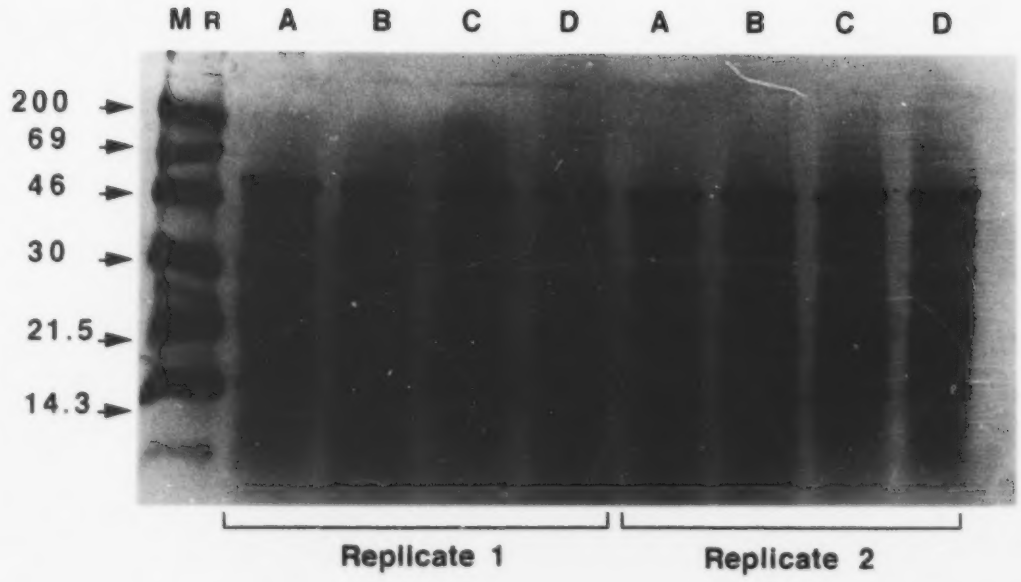

MR A B C D A B C D
200
69
46
30
21.5
14.3
Replicate 1
Replicate 2

Figure 4c Northern blot (top right panel) showing the expression of *lsu* in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Northern blot.

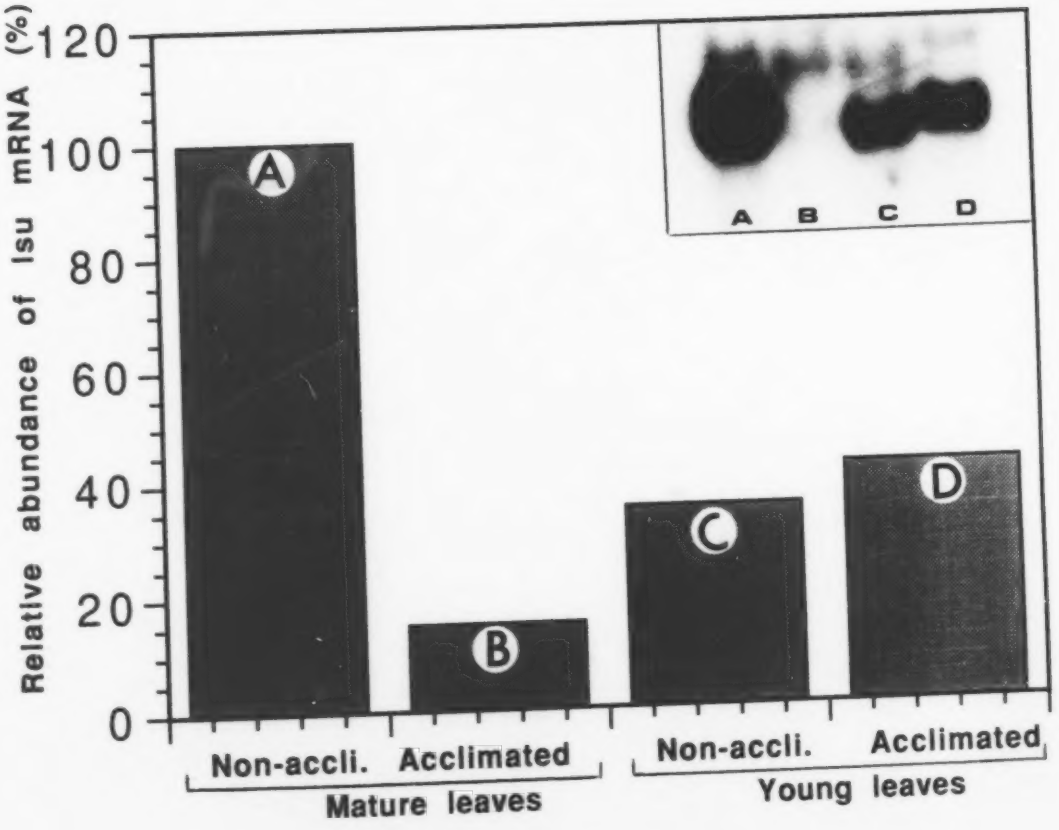

Relative abundance of Isu mRNA (%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Mature leaves
Non-accli.
Acclimated
Young leaves

Figure 4d Western blot (top right panel) showing the relative accumulation of LSU polypeptide in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Western blot.

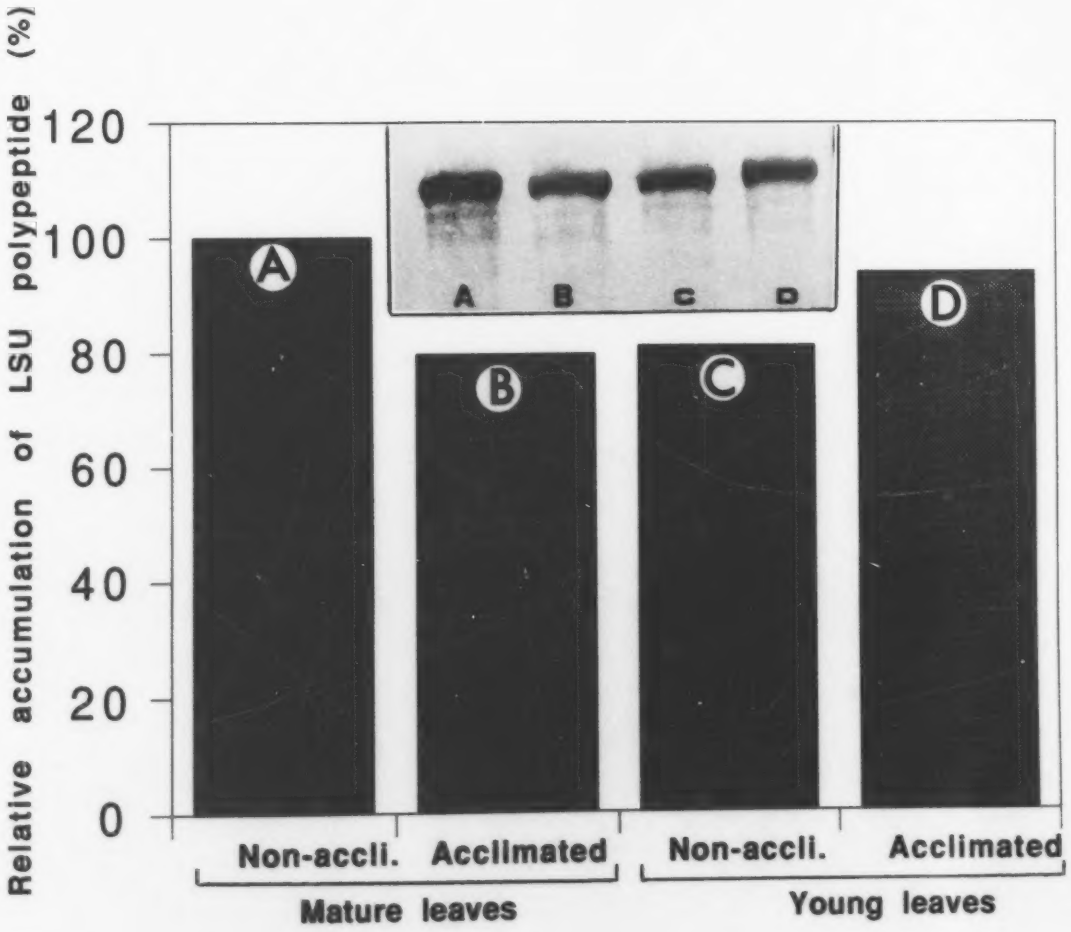

Relative accumulation of LSU polypeptide (%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Non-accli.
Acclimated
Mature leaves
Young leaves

Figure 4e Northern blot (top right panel) showing the expression of *ssu* in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Northern blot.

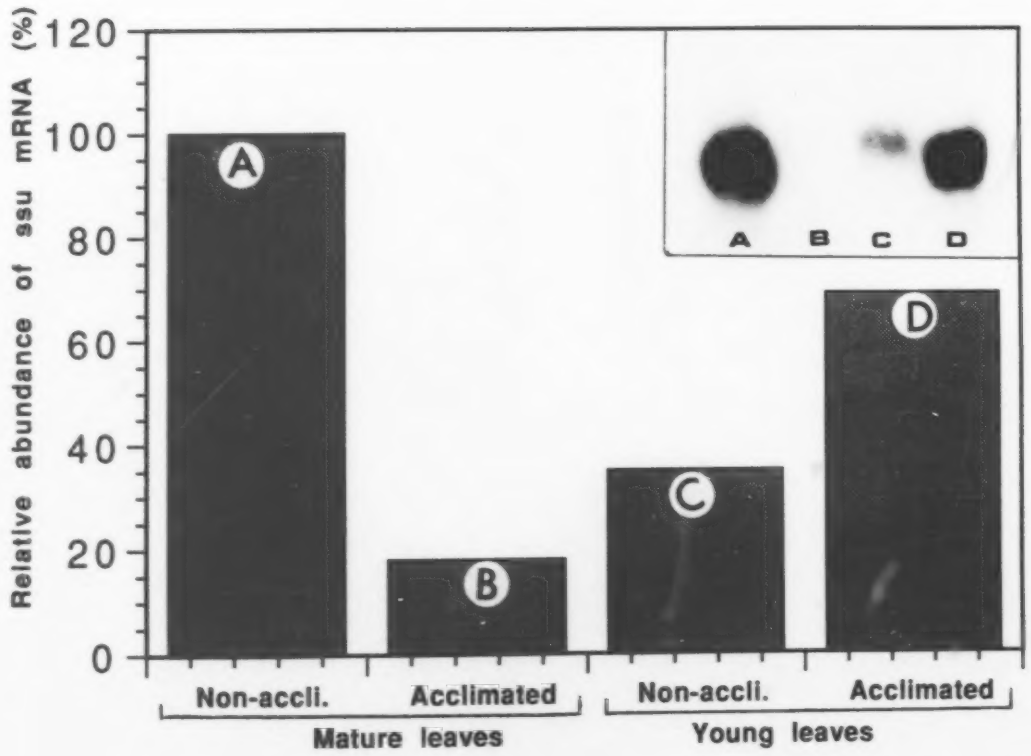

Relative abundance of ssu mRNA (%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Non-accli.
Acclimated
Mature leaves
Young leaves

Figure 4f Western blot (top right panel) showing relative accumulation of SSU polypeptide in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Western blot.

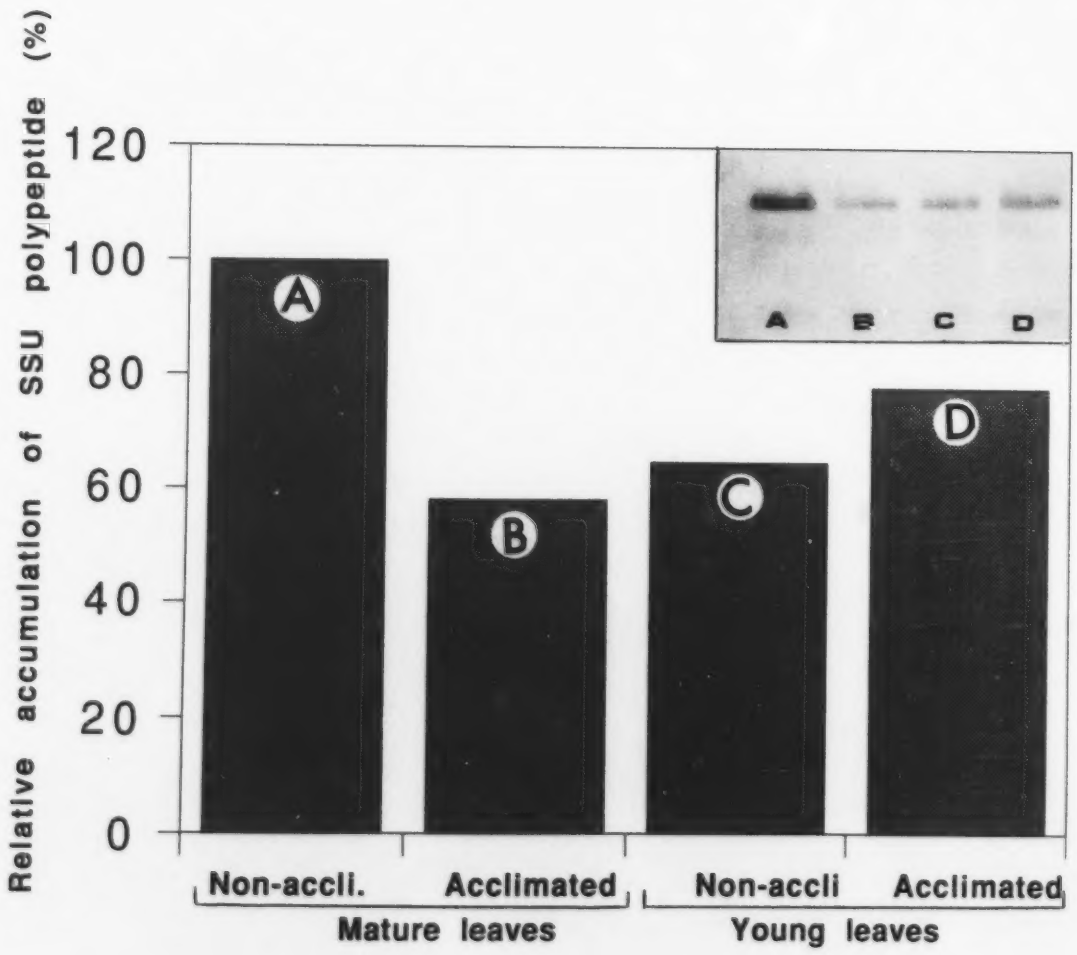

Relative accumulation of SSU polypeptide (%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Non-accli
Acclimated
Mature leaves
Young leaves

Figure 4g Northern blot (top right panel) showing the expression of *cab* in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Northern blot.

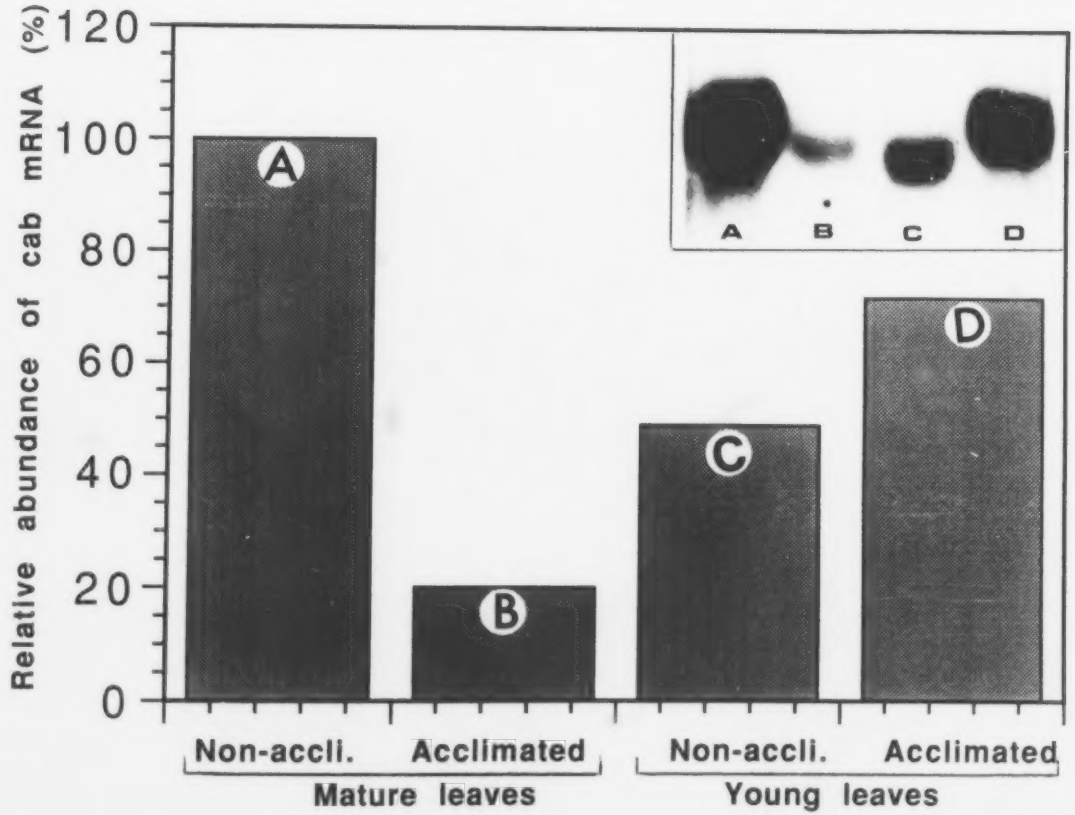

Relative abundance of cab mRNA (%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Non-accli.
Acclimated
Mature leaves
Young leaves

Figure 4h Western blot (top right panel) showing relative accumulation of CAB polypeptides in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Western blot.

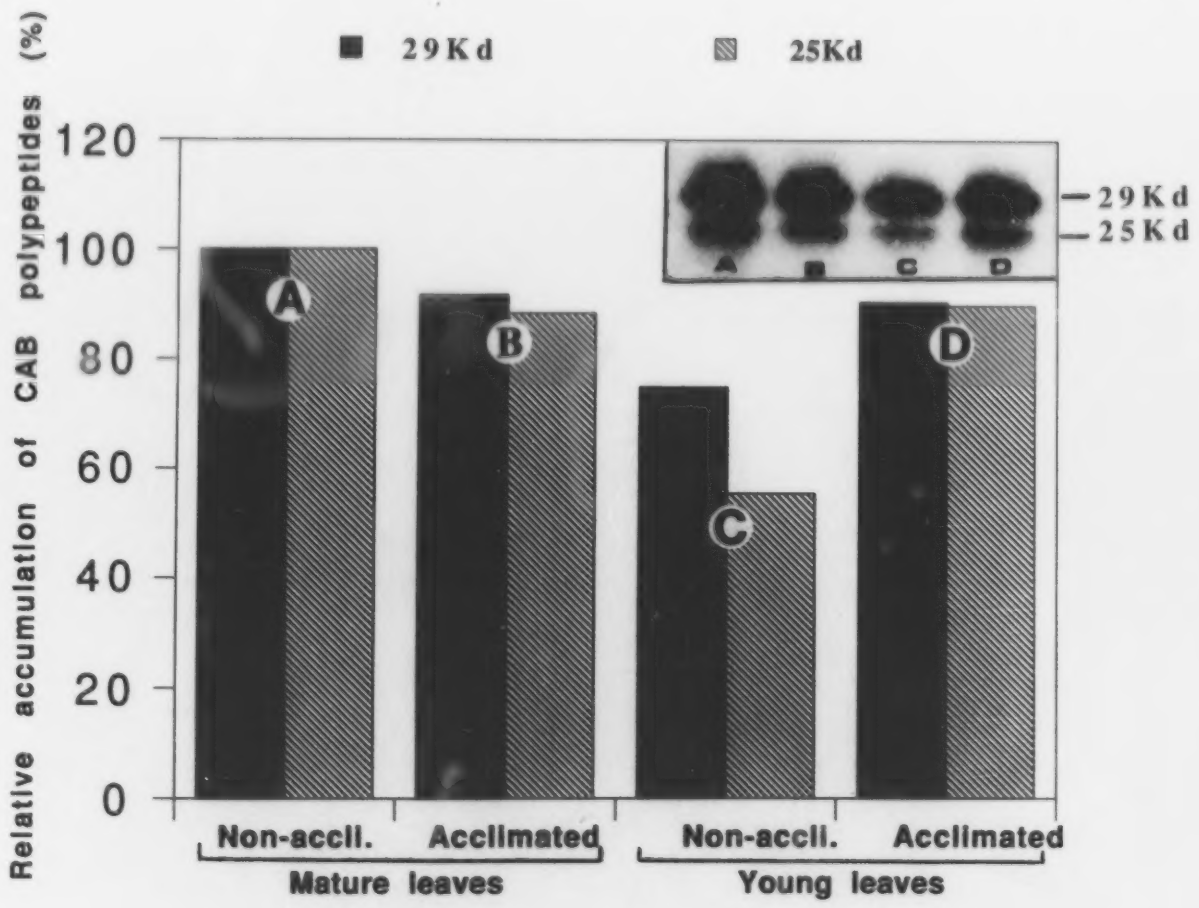

29Kd
25Kd
Relative accumulation of CAB polypeptides (%)
120
100
80
60
40
20
0
A
B
C
D
29Kd
25Kd
Non-accli.
Acclimated
Non-accli.
Acclimated
Mature leaves
Young leaves

Figure 4I Western blot (top right panel) showing relative accumulation of CAB polypeptides. Total protein was loaded on the basis of equal chlorophyll content in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Western blot.

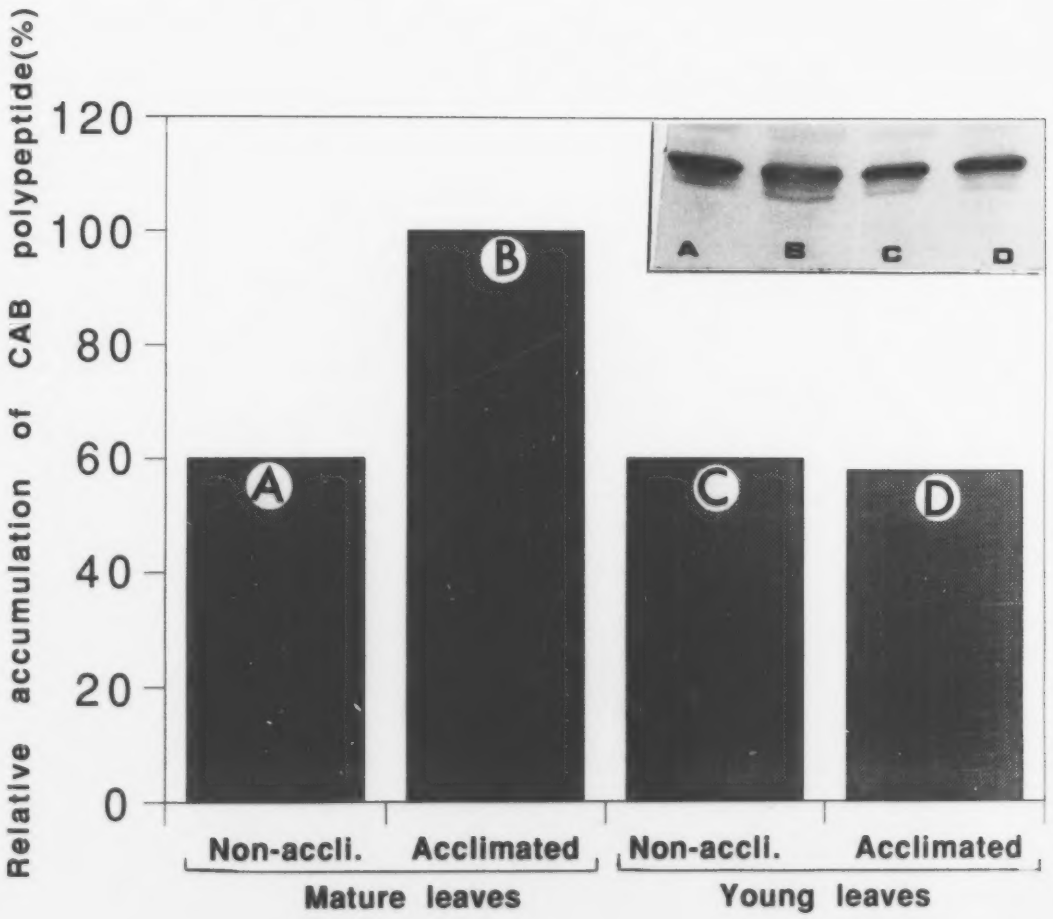

Relative accumulation of CAB polypeptide(%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Non-accli.
Acclimated
Mature leaves
Young leaves

Figure 4j Northern blot (top right panel) showing the expression of *psbA* in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Northern blot.

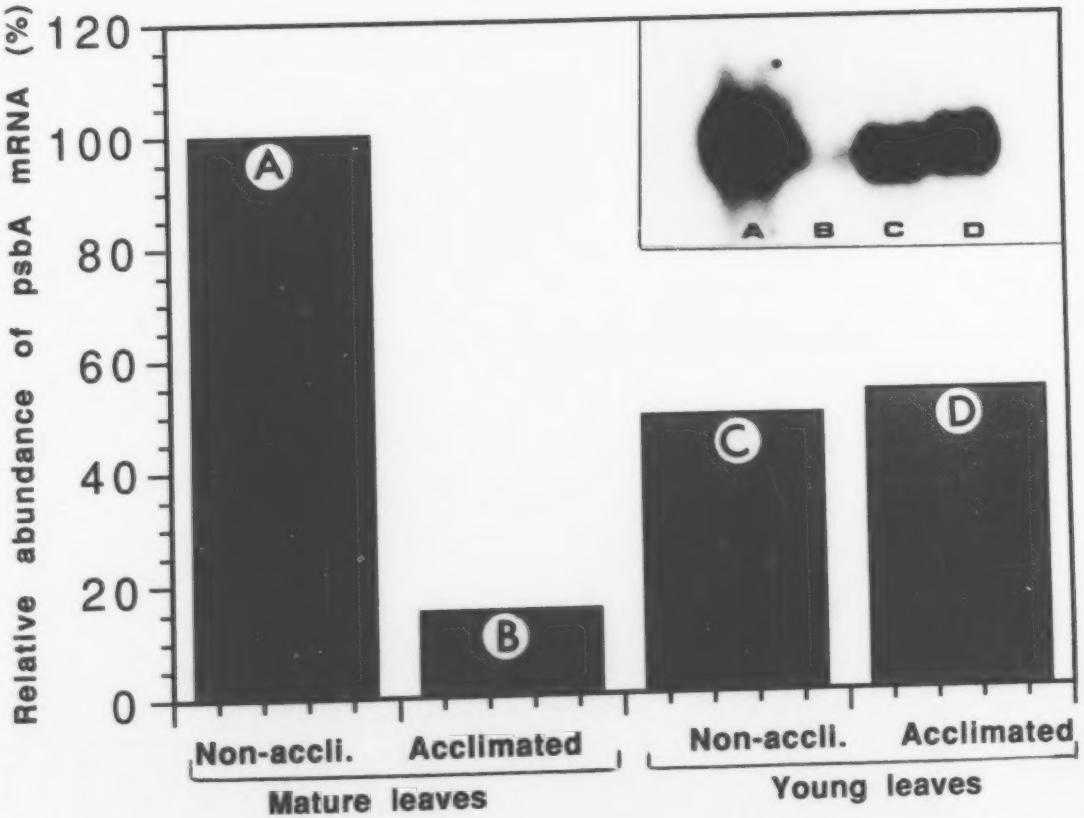

Relative abundance of psbA mRNA (%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Non-accli.
Acclimated
Mature leaves
Young leaves

Figure 4k Western blot (top right panel) showing relative accumulation of PSBA polypeptide in *Brassica napus* leaves during low temperature acclimation. The histogram below represents the densitometry scan of the Western blot.

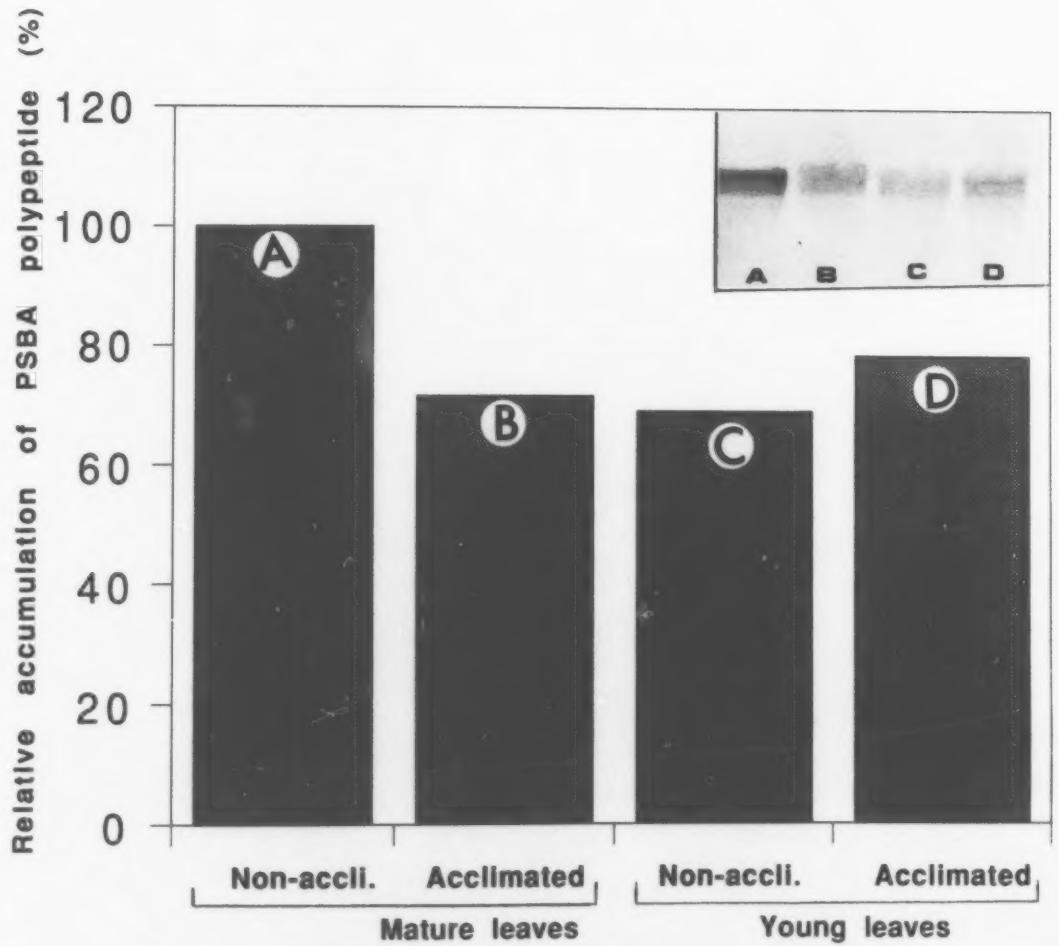

Relative accumulation of PSBA polypeptide (%)
120
100
80
60
40
20
0
A
B
C
D
A B C D
Non-accli.
Acclimated
Non-accli.
Acclimated
Mature leaves
Young leaves

Figure 41 An example of an autoradiogram showing the kinetics of protein turn over in *Brassica napus* leaves with 60 minutes pulse and 10hr chase periods. Each lane was loaded on the basis of equal radioactive counts (100,000 c.p.m.).

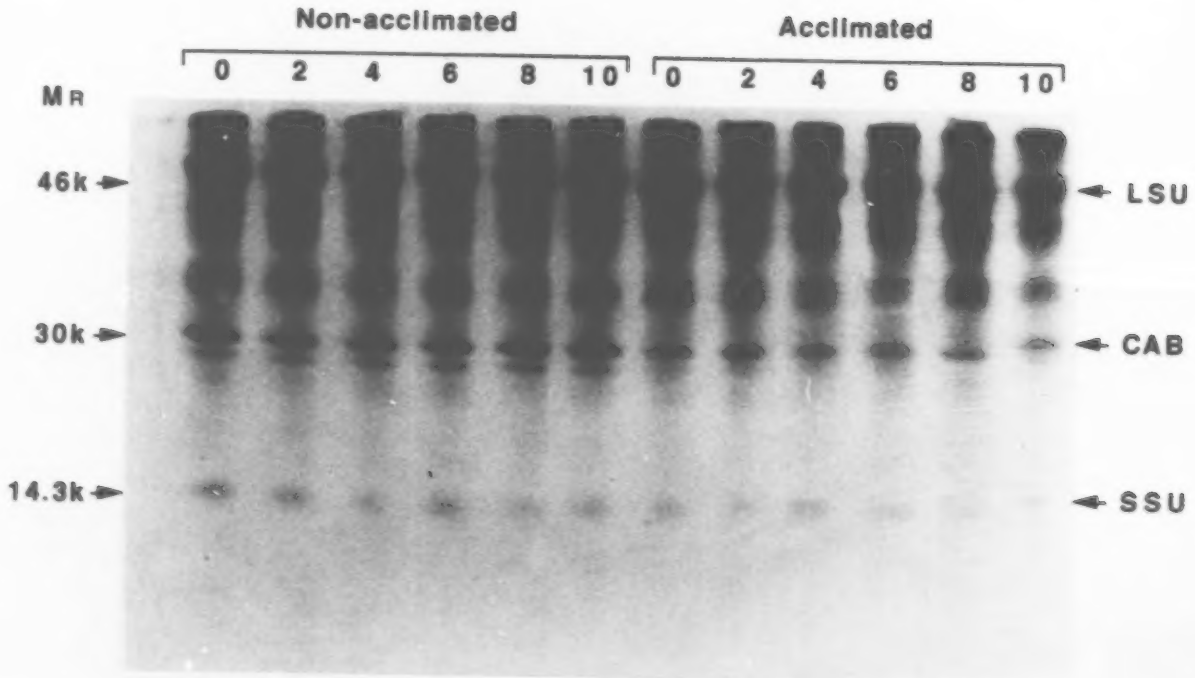
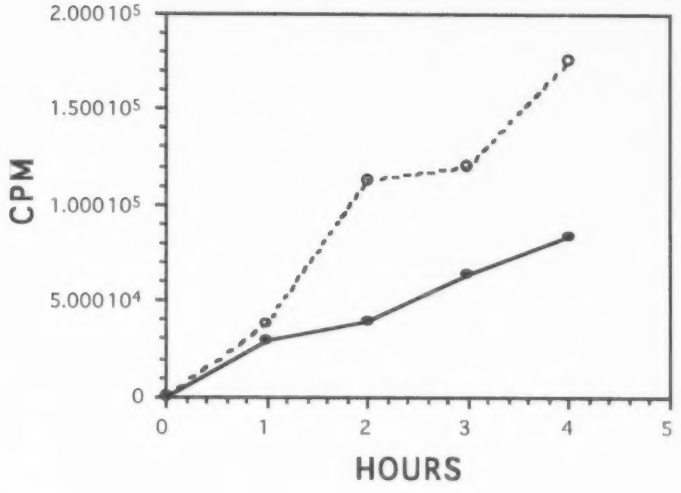
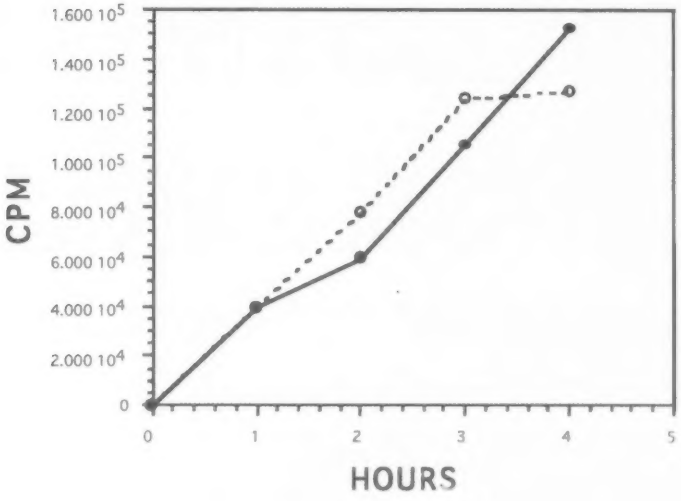

Non-acclimated
Acclimated
0 2 4 6 8 10
0 2 4 6 8 10
MR
46k
30k
14.3k
LSU
CAB
SSU

Figure 4m Incorporation of ^{35}S methionine into protein in non-acclimated and acclimated mature and young leaves of *Brassica napus* during 4hrs of labeling. Open circles represent Non-acclimated and closed circles represent acclimated.

^{35}S-methionine incorporation into protein in mature leaves



^{35}S-methionine incorporation into protein in young leaves



CHAPTER 5

Methylation studies on photosynthetic genes during low temperature acclimation in *Brassica napus*

INTRODUCTION

Transcriptional regulation of gene expression involves a complex, multifaceted process that includes interactions of many trans-acting factors with specific cis-acting DNA sequences. Selective methylation of DNA provides one mechanism for altering the local structure of a gene, and, indeed, various methylation patterns of higher plant nuclear DNA have been reported (Hepburn *et al*, 1987; Gruenbaum *et al*, 1981; Scott *et al*, 1984; Von Kalm *et al*, 1986). DNA methylation causes the repression of gene activity as indicated by studies showing that the transcription of *in vitro* methylated DNA sequences was inhibited when inserted into various cell types in culture.

Studies of gene methylation in plants have shown that hypomethylation is associated with high rates of transcription in a number of genes, ranging from rDNA to photosynthetic genes (Kobayashi *et al*, 1990; Ngernprasirtsiri *et al*, 1988a; 1988b; 1989; Watson *et al*, 1987). Low transcription rates have been correlated with DNA methylation in plastids during the transition of chloroplasts to chromoplasts in tomato (Kobayashi *et al*, 1990). Hypomethylation is correlated with high transcript abundance and hypermethylation is correlated with low transcript abundance. For example, *lsu* and *ssu* expression is correlated with hypomethylation in bundle sheath cells, whereas, in mesophyll cells, the absence of *lsu* and *ssu* expression is correlated with hypermethyaltion of these genes. Further, a heterologous *in vitro* transcription system using *E. coli* RNA polymerase revealed that *lsu* in mesophyll and bundle sheath cells was active as a template in the hypomethylated state and inactive in the hypermethylated state (Kobayashi *et al*, 1990). Unfortunately, hypomethylation is not always associated with high transcript levels. For example, Ngernprasirtsiri *et al*, (1988 a, b) showed hypermethylation of *ppc* (phosphoenol pyruvate carboxylase) and *pdk* (orthophosphate dikinase) which are nuclear encoded genes, in mesophyll

cells correlated to high transcript abundance and hypomethylation in bundle sheath cells correlated with low transcript abundance. In contrast, results from *in vitro* transcription assays showed a positive correlation between hypermethylation and low transcript levels.

There are no reports regarding the relationship between methylation and developmental regulation of photosynthetic gene expression with regard to tissue age or acclimation. Results from previous work (Chapter 4) indicate that *lsu, ssu, cab* and *psbA* are differentially expressed during acclimation in young and mature leaves. The objective of the present study was to investigate selective methylation of these genes. To this end, *Hind III, Eco RI, Pst I, Msp I* and *Hpa II* were used in restriction digests of chloroplast and total genomic DNA.

MATERIALS AND METHODS

Plant material

Growth conditions and harvesting of leaves followed the protocols outlined in Chapter 3.

Isolation of total plant DNA and chloroplast DNA

Total genomic DNA and chloroplastic DNA were isolated according to the protocol in Chapter 3.

Restriction of total genomic DNA and chloroplastic DNA

Restriction protocols were the same as in Chapter 3. The enzymes used for the methylation studies were *EcoRI, Hind III, Pst I, Msp I* and *Hpa II.*

Probes

psbA and *lsu* inserts were random primed with α-^{32}P-dCTP and probed for hybridization pattern differences in chloroplastic DNA. *cab* and *ssu* inserts were random primed with α-^{32}P-dCTP and probed for hybridization differences in total genomic DNA.

Hybridization

Hybridization and washing conditions were the same as in Chapter 3.

Densitometry scan

Hybridization signals for ssu and cab had high backgrounds and were, therefore, scanned. A GS-670 Imaging densitometer (Biorad Image Analysis Sytem) was used and the relative mobility of each peak was recorded. The results were then expressed diagrammatically. An example of such a Southern blot is shown in Fig 5a.

RESULTS

Acclimation in mature leaves was generally associated with decreased methylation of *ssu*, as indicated by the increase in the number of restriction fragments in *Eco RI, Hind III, Pst I* and *Hpa II* digests (Fig. 5b and 5c). In addition, differences in the *Msp I* restriction fragments were noted between the non-acclimated and acclimated mature leaves (Fig. 5c). On the other hand, acclimation in young leaves did not appear to be associated with changes in the overall amount of methylation, as *Eco RI* and *Pst I* digests produced 1 less fragment each, *Hind III* and *Hpa II* had 1 more fragment each and *Msp I* digests had 3 more fragments in the acclimated young leaves. Comparisons between the young and mature leaves show generally higher numbers of restriction fragments in the young leaves, indicating lower methylation at these sites.

In mature leaves, methylation of *cab* decreased with acclimation. Increases in the number of restriction fragments were noted for all enzymes, except *Pst I*, which showed no change in the number of fragments (Fig. 5e). Although *Eco RI, HindIII, Hpa II* and *Msp I* detected differences in methylation with acclimation in the young leaves, there was little changes overall (Fig. 5d and 5e). Again *Pst I* digests failed to show any change in the restriction pattern with acclimation. In both mature and young leaves, *Msp I* detected differences in methylation between the acclimated and non-acclimated state. Comparisons between the two leaf ages showed that the *cab* genes in the young leaves had fewer methylated sites than in the mature leaves.

Both mature and young leaves showed decreased methylation of *lsu* and *psbA* with acclimation. Southern blots that were probed for *lsu* and *psbA* showed no differences in the *Eco RI* hybridization patterns (Fig. 5f). The presence of an additional band in *Hind III* digests of *lsu* from acclimated young leaves indicate that either the 5' C or A was hypomethylated at AAGCTT (Fig. 5g). This difference was not seen in the mature leaves. Hypomethylation at CTGCAG sequence of *lsu* was seen with *Pst I* digestion during low temperature acclimation in both mature and young leaves (Fig. 5h). Hybridization differences were also seen in *psbA* at *Hind III* sites (Fig. 5g). Higher accessibility, (indicated by asterisk at high intensity hybridization signal at about 5 kb) was noted in acclimated young leaves (Fig. 5g). The same site was less accessible to *Hind III* restriction in acclimated mature leaves. *Msp I* and *Hpa II* restrictions did not reveal any methylation differences in *lsu* or *psbA* genes (Fig. 5i and 5j). However, *Hpa II* restrictions showed hybridization signals of larger DNA fragments as compared to *Msp I* restrictions thereby indicating that the internal cytosine residues were methylated in both *lsu* and *psbA* genes (Fig 5i and Fig 5j).

DISCUSSION

In the present study, differences in methylation were noted between both acclimated and non-acclimated mature and young leaves. These differences were both qualitative and quantitative. In general, the quantity of methylation (based on number of fragments) decreased with acclimation in both leaf ages and was higher in mature leaves.

Changes in methylation status could be correlated with acclimation. For example, hypomethylation of *Hpa II* sites of *ssu*, *Hind III* sites of *ssu*, *Eco R I* sites of *cab* and *Pst I* sites of *lsu* and *psbA* correlated with acclimation. Similar relationships could be seen between methylation and leaf age. Hypomethylation of the *Eco R I*, *Hind III* and *Hpa II* sites of *ssu* and *Pst I*, *Hpa II* , *Msp I* sites of *cab* and *Pst I* sites of *lsu* was found in the young leaves.

The relationship between gene methylation and transcription rates is not clear. However, it is generally accepted that gene activity is associated with hypomethylation. In a previous study (Chapter 4), *ssu*, *cab*, *lsu* and *psb A* gene expression was shown to decrease dramatically with acclimation in mature leaves. On the other hand, in young leaves, expression of *ssu* and *cab*

increased markedly with acclimation, while *lsu* and *psbA* underwent less pronounced increases. These changes were all associated with hypomethylation (Fig. 5b, c, d, e and g). Similarly, less methylation in young leaves relative to that in mature leaves was associated with lower transcript levels. Thus, it would appear that methylation was associated with high transcript levels in mature leaves and lower transcript levels in young leaves. There are a number of possible explanations for these differences including : only methylation of specific sequences is important in gene control; message stability is low in mature leaves; and there is a threshold level of methylation, above which changes in status fail to affect gene transcription.

Certainly, other workers have found variations in methylation associated with increases or decreases in transcript levels. For example, in non-photosynthetic cells of amyloplasts of *Sycamore*, hypermethylation correlated to low expression of *lsu, psaA, atp A, B* and *E*, and rps4 genes (Ngernprasirtsiri *et al*, 1988a). Methylation was not detected in *psbA* and 16S rRNA genes and this correlated to high expression of the respective genes (Ngernprasirtsiri *et al*, 1988a). During tomato fruit development, methylation at *Hpa II* sites were noted in *lsu, psaA, atp E* and *rpS4* genes and this correlated with low expression of the genes in *in vitro* transcription assays (Ngernprasirtsiri *et al*, 1988b). Ngernprasirtsiri *et al*, (1989) showed low expression of *lsu* and *ssu* correlated with hypermethylation at *Eco R II* sites. In contrast, the same group (Ngernprasirtsiri *et al*, 1989) showed high expression of *ppc* and *pdk* correlated with hypermethylation in bundle sheath cells. Furthermore, their *in vitro* transcription studies showed that expression of *lsu* correlated with the hyper and hypo methylation status in bundle sheath and mesophyll cells of maize leaves respectively. Nuclear-run on transcription assays and *in vitro* transcriptions assays have revealed a direct correlation of low expression of *lsu, psa A, atp B, E* and *rps 4* genes in chromoplasts with hypermethylation (Kobayashi *et al*, 1990).

If changes in methylation do control transcript levels, this should be apparent in comparisons between acclimated and non-acclimated leaves and between young and mature leaves. Examination of *ssu* shows a very loose correlation between hypermethylation at *Pst I* sites and higher transcript levels. Similarly, a loose correlation was apparent between methylation at *Hind III* sites and *cab* levels. Although decreased methylation at *Pst I* sites in

lsu could be correlated with acclimation, there was no correlation between methylation and gene expression. Finally, *psb A* methylation could not be correlated with gene expression related to acclimation or leaf age. Thus, if there was any relationship between methylation and transcript levels, it was a positive correlation and was restricted to nuclear encoded genes.

In conclusion, results from this study show changes in methylation in both nuclear and chloroplast encoded genes. Such changes in methylation could be correlated with changes in transcript levels, but these correlations were weak and were positive, i.e. increased methylation was associated with increased transcript levels. The overall trends were less clear, as methylation was higher and transcript levels were both high and low in mature leaves relative to the young leaves. Thus, although hypomethylation could be correlated with acclimation and was more prevalent in young leaves, the significance of these correlations is dubious.

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Watson J.C.; Kaufman L.S. and Thompson W.F. 1987. Developmental regulation of cytosine methylation in the nuclear ribosomal RNA genes of Pisum sativum. J Mol Biol 193: 15-26.

Fig 5a An example of a Southern blot analysis of *cab* showing the hybridization signals with high background in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Total genomic DNA was restricted with *Msp I, Hpa II* and *Pst I* enzymes. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

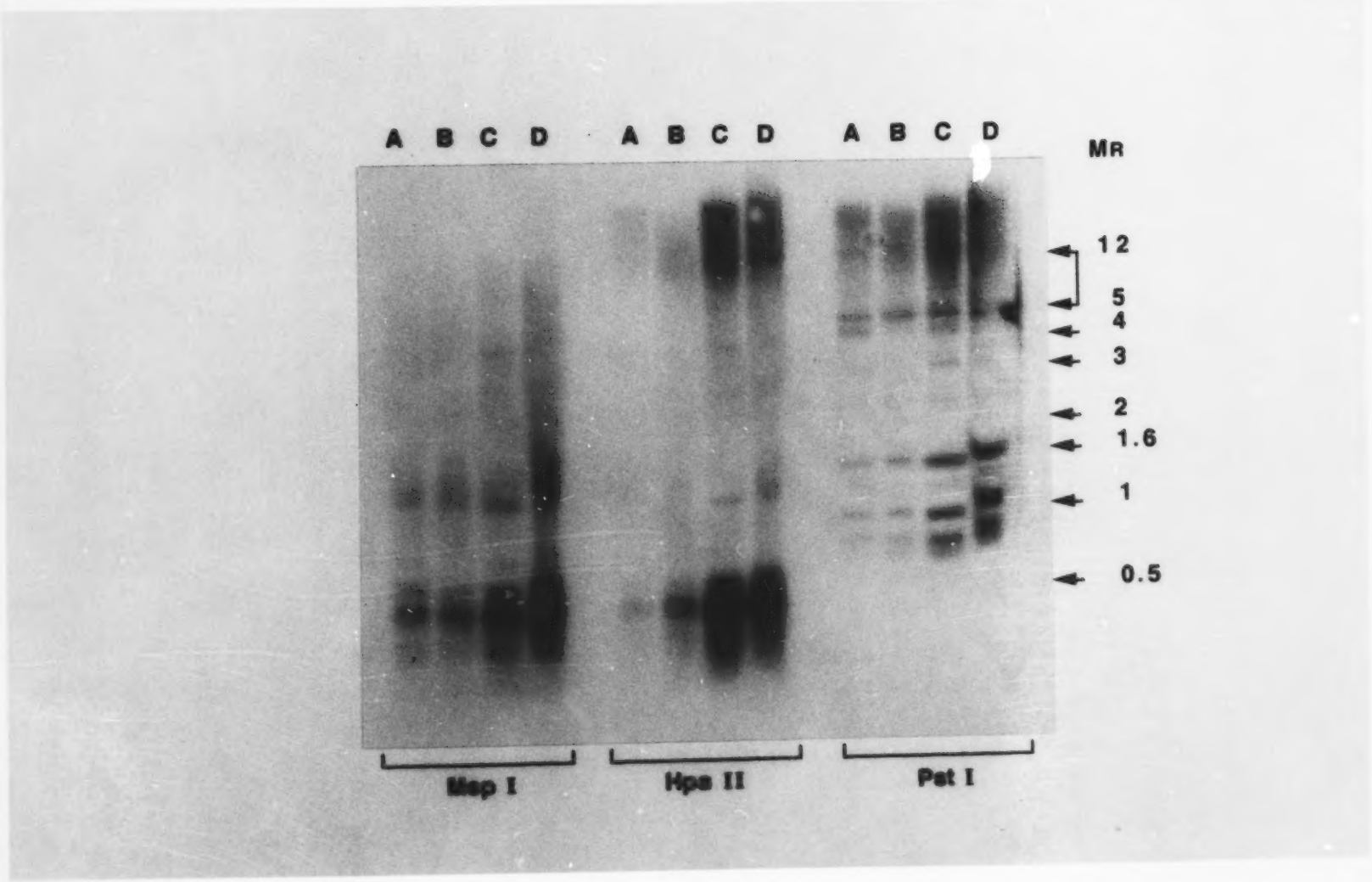

A B C D A B C D A B C D
MR
12
5
4
3
2
1.6
1
0.5
Msp I
Hpa II
Pst I

Figure 5b Diagramatic representation of Southern analysis, showing the hybridization patterns of *ssu* in *Eco RI* and *Hind III* restriction of total genomic DNA in mature and young leaves of *Brassica napus* during low temperature acclimation. Differences are indicated by asterisk. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

Hybridization patterns of *ssu*

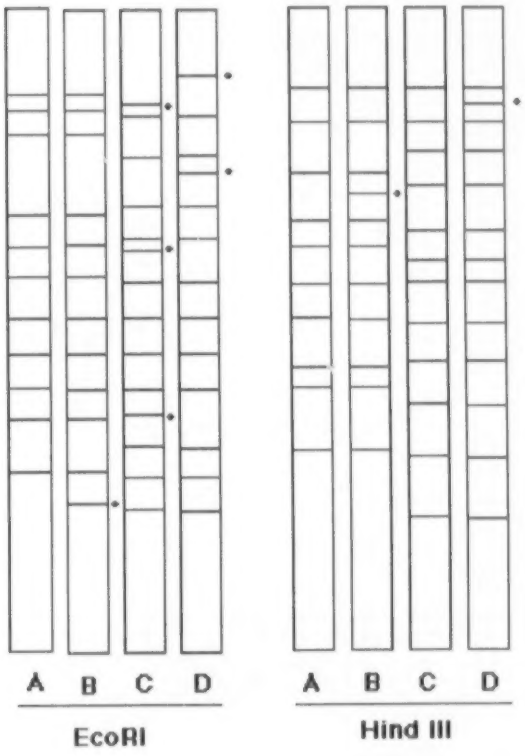


Figure 5c Diagramatic representation of Southern analysis showing the hybridization patterns of *ssu* in *Pst I, Hpa II* and *Msp I* restriction of total genomic DNA in mature and young leaves of *Brassica napus* during low temperature acclimation. Differences are indicated by asterisk. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

Hybridization patterns of *ssu*

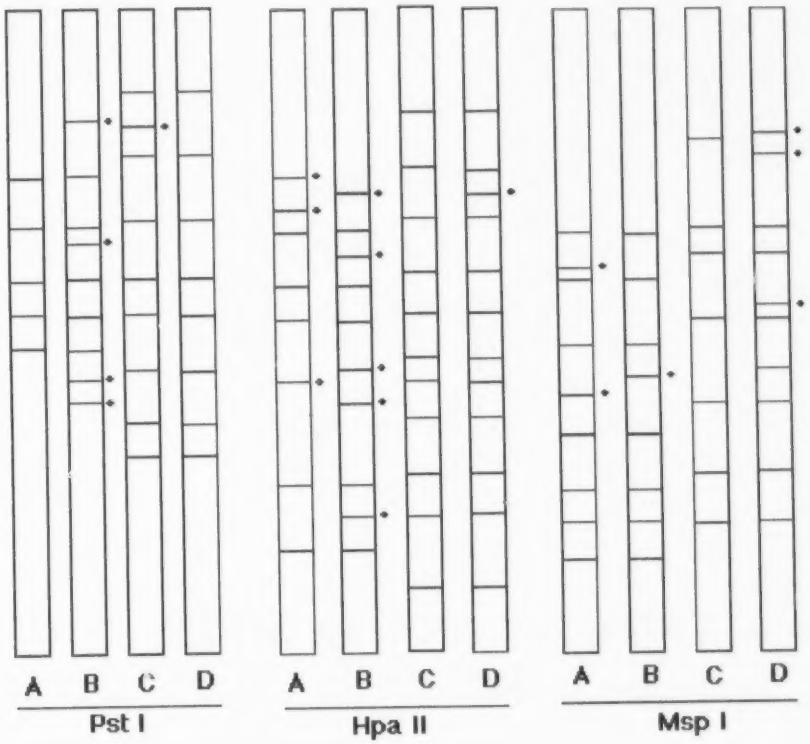


Figure 5d Diagramatic representation of Southern analysis showing the hybridization patterns of *cab* in *Eco RI* and *Hind III* restriction of total genomic DNA in mature and young leaves of *Brassica napus* during low temperature acclimation. Differences are indicated by asterisk. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

Hybridization patterns of *cab*

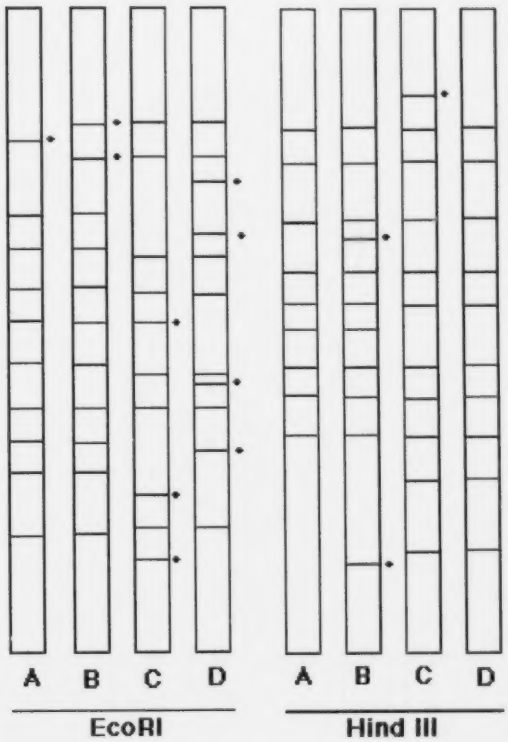


Figure 5e Diagramatic representation of Southern analysis showing the hybridization patterns of *cab* in *Pst I, Hpa II* and *Msp I* restriction of total genomic DNA in mature and young leaves of *Brassica napus* during low temperature acclimation. Differences are indicated by asterisk. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

Hybridization patterns of *cab*

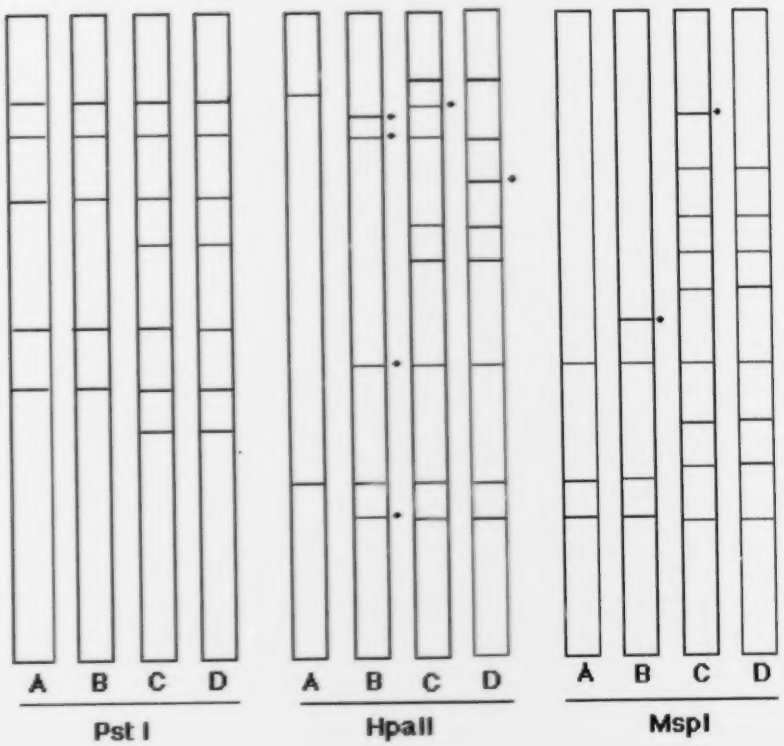


Figure 5f Southern analysis showing the hybridization patterns of chloroplastic rDNA (11-5), *lsu,* and *psb A* in *Eco RI* restriction digests of chloroplastic DNA in mature and young leaves of *Brassica napus* during low temperature acclimation. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

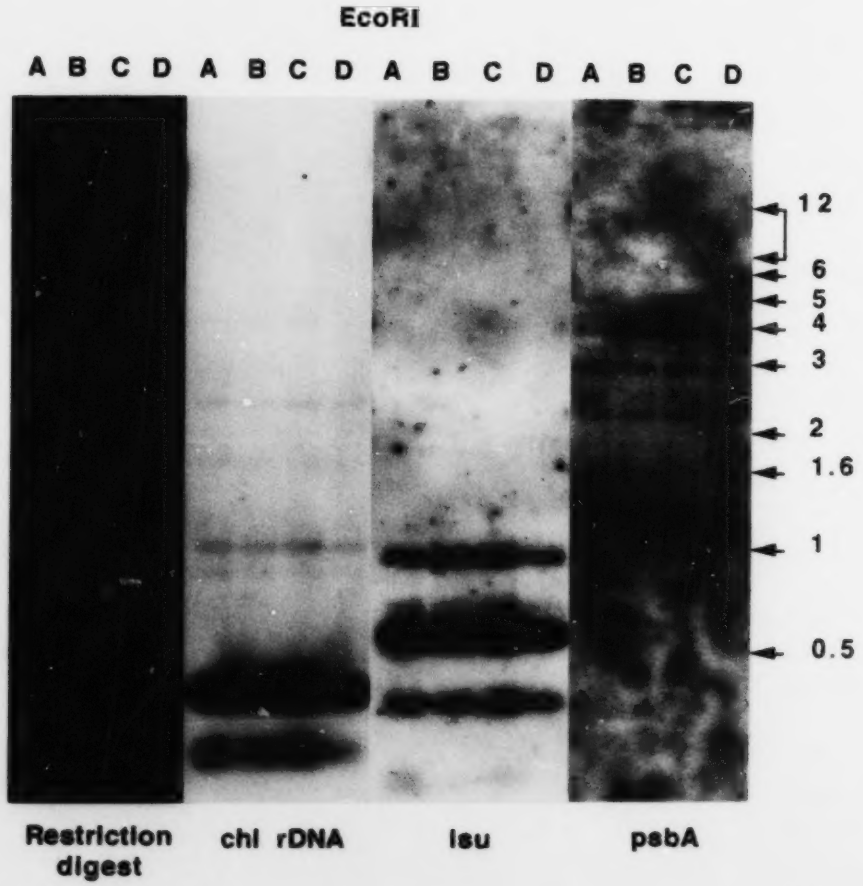

EcoRI
A B C D A B C D A B C D A B C D
12
6
5
4
3
2
1.6
1
0.5
Restriction digest
chl rDNA
lsu
psbA

Figure 5g Southern analysis showing the hybridization patterns of chloroplastic rDNA (11-5), *lsu,* and *psb A* in *Hind III* restriction digests of chloroplastic DNA in mature and young leaves of *Brassica napus* during low temperature acclimation. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

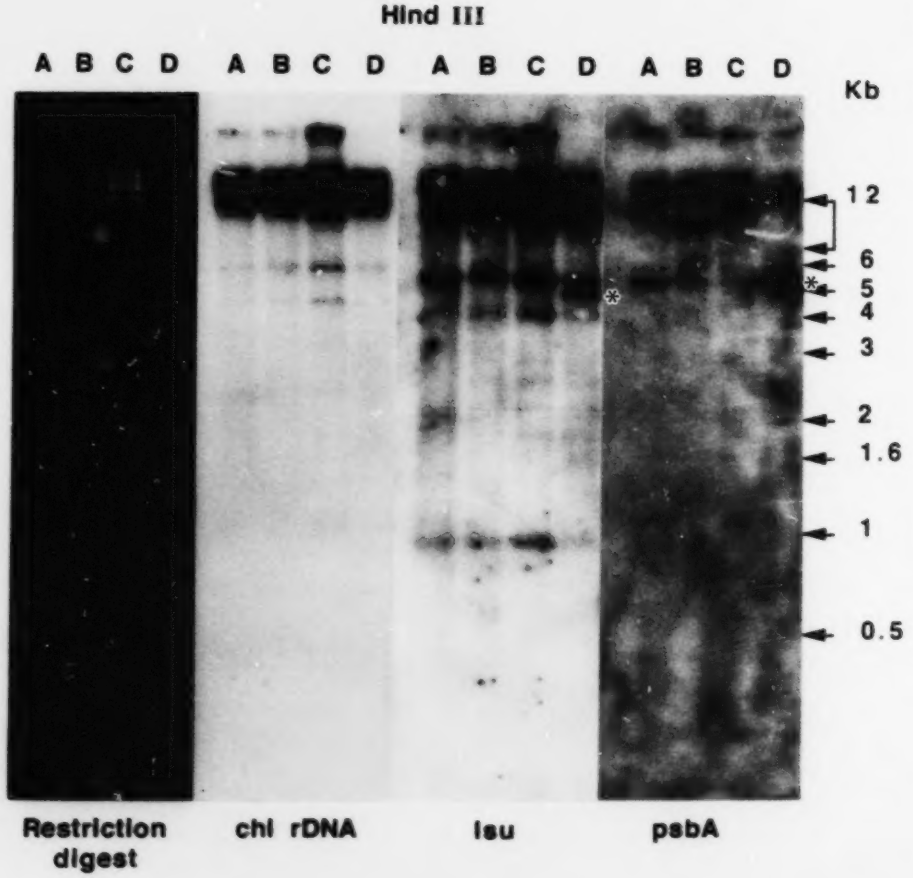

Hind III
A B C D A B C D A B C D A B C D
Kb
12
6
5
4
3
2
1.6
1
0.5
Restriction digest
chl rDNA
lsu
psbA

Figure 5h Southern analysis showing the hybridization patterns of chloroplastic rDNA (11-5), *lsu*, and *psb A* in *Pst I* restriction digests of chloroplastic DNA in mature and young leaves of *Brassica napus* during low temperature acclimation. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

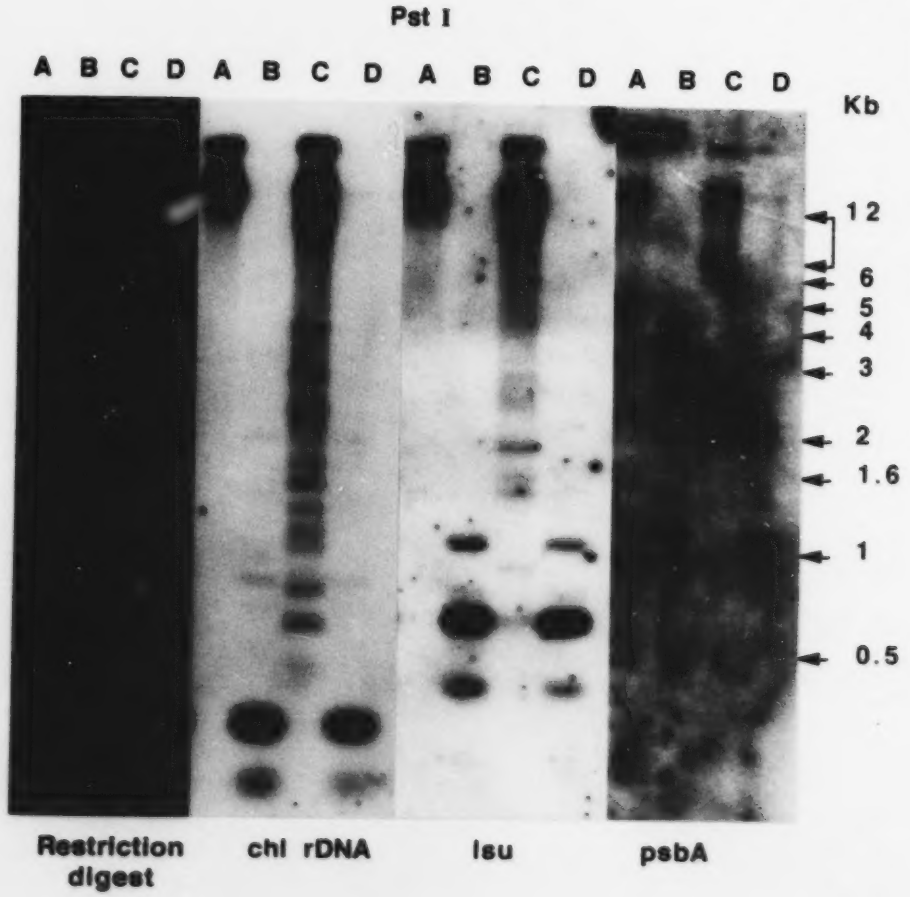

Pst I
A B C D A B C D A B C D A B C D
Kb
12
6
5
4
3
2
1.6
1
0.5
Restriction digest
chl rDNA
lsu
psbA

Figure 5i Southern analysis of *lsu* in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Restriction digests of *Msp I* and *Hpa II* (left) and the hybridization signals of *lsu* (right) are shown. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.

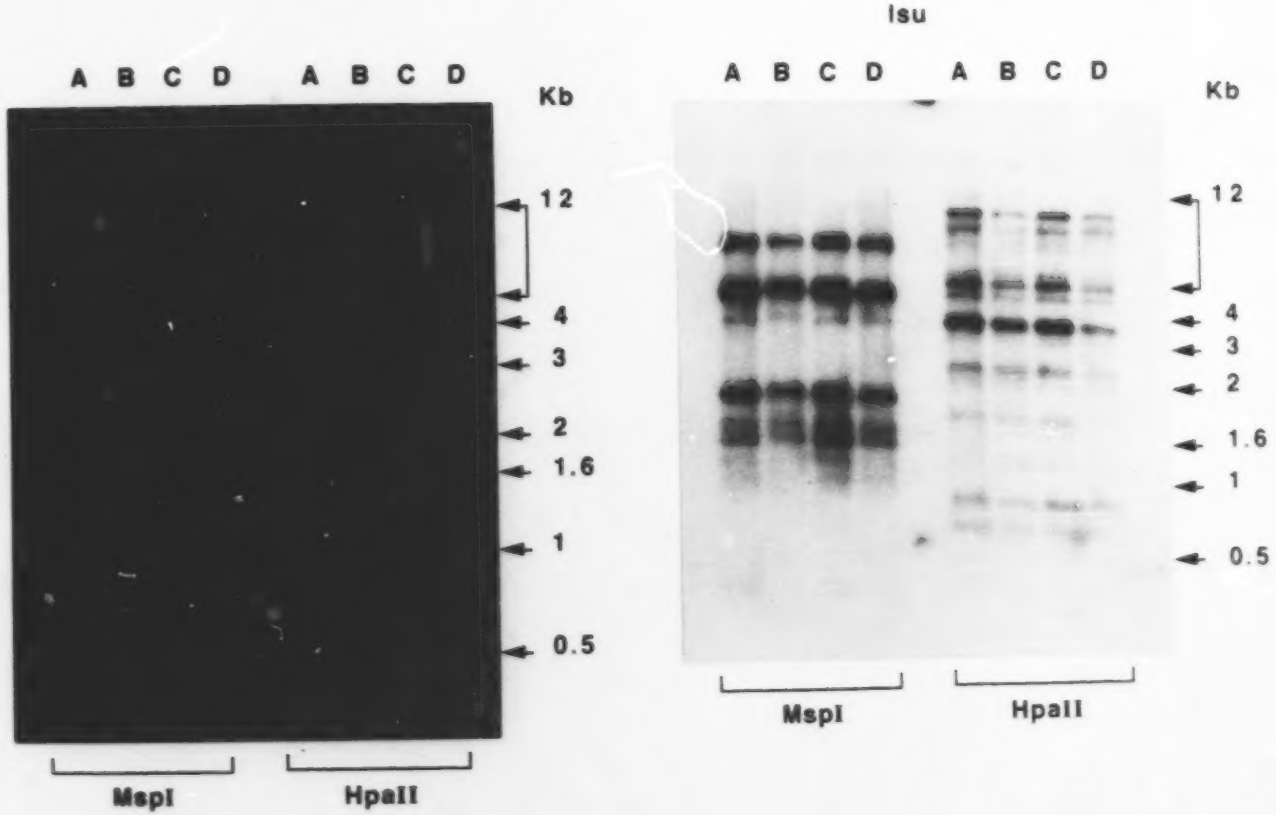
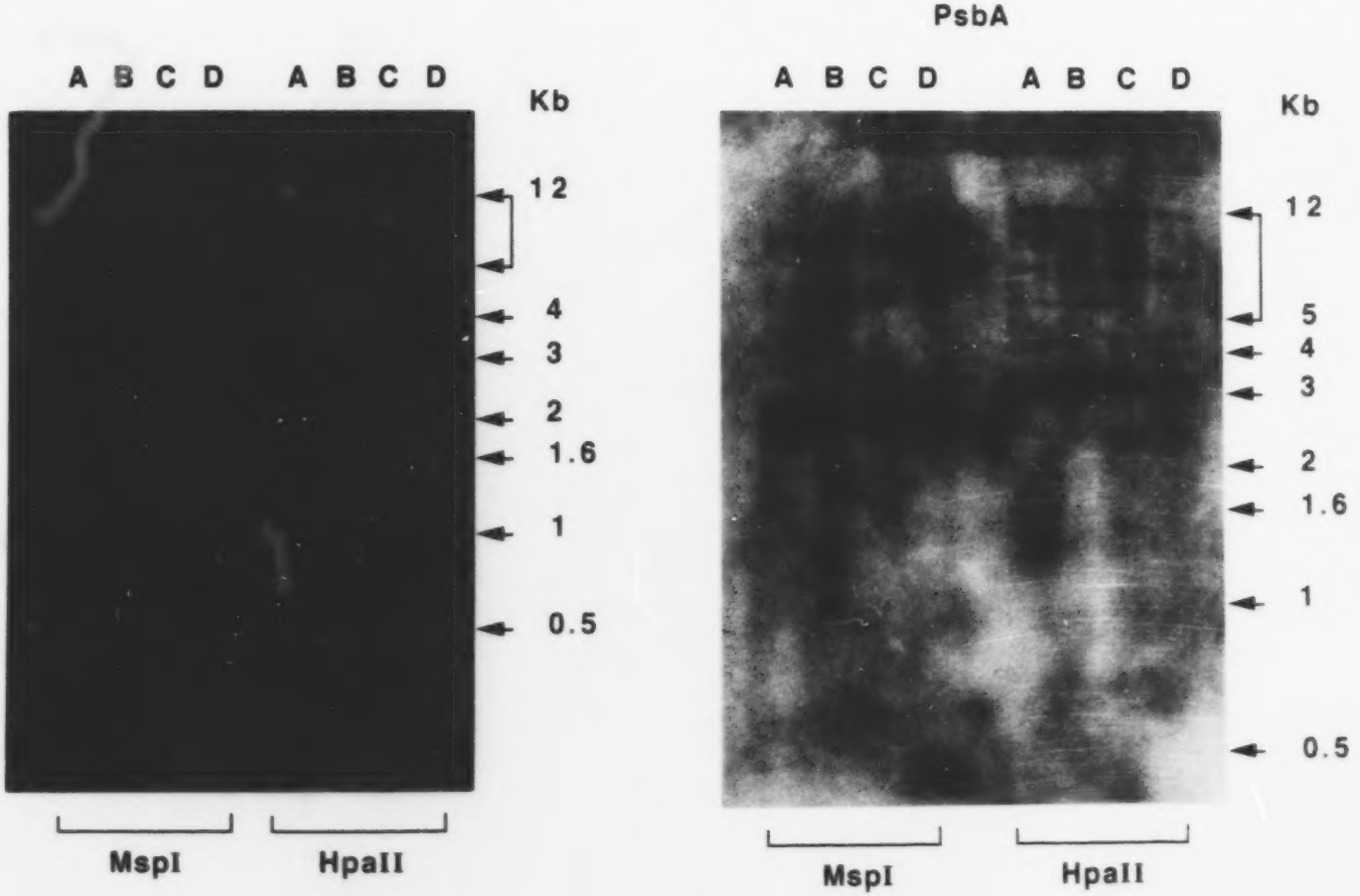

Isu
A B C D A B C D
Kb
12
4
3
2
1.6
1
0.5
MspI
HpaII
A B C D A B C D
Kb
12
4
3
2
1.6
1
0.5
MspI
HpaII

Figure 5j Southern analysis of *psbA* in mature and young leaves of *Brassica napus* during 3 weeks of low temperature acclimation. Restriction digests of *Msp I* and *Hpa II* (left) and the hybridization signals of *psbA* (right) are shown. A-non-acclimated mature leaves, B-acclimated mature leaves, C-non-acclimated young leaves and D-acclimated young leaves.


PsbA
A B C D A B C D
Kb
12
4
3
2
1.6
1
0.5
MspI
HpaII
A B C D A B C D
Kb
12
5
4
3
2
1.6
1
0.5
MspI
HpaII

CHAPTER 6

SUMMARY

One of the most fundamental metabolic process in the plant is photosynthesis. It provides the carbon skeleton for growth and development and thus any alteration of photosynthetic function will have a large affect on the plant. It is known that low temperature limits plant growth, but, there are many plants including winter annuals and perennials that adjust to low temperature. This process is referred to as low temperature acclimation. It includes adjustment of photosynthesis. While much is known about the response of photosynthesis to low temperature acclimation, little is known about the regulation photosynthetic gene expression. This thesis addresses how the plant responsds to low temperature acclimation by adjusting expression of the photosynthetic genes and their protein products, including the role of gene methylation on the photosynthetic gene expression.

Two different leaf ages were used in the present study to understand the effect of low temperature acclimation on photosynthetic gene expression in *Brasscia napus* cv. Jet Neuf. *Brassica napus* cv. Jet Neuf was used, as this plant is a winter cultivar and can acclimate to low temperatures. The four separate studies that were carried out on mature and young leaves strongly suggest that it is extremely important to define tissue type before attempting to gain any information on low temperature acclimation. Extensive studies have clearly shown that growth and development differ amongst leaf ages, yet no studies have been undertaken to study the effect of developmental differences on freezing tolerance.

During low temperature acclimation both mature and young leaves showed increased freezing tolerance with time. Relative freezing tolerance of both the leaves were similar in the non-acclimated state but differed as the leaves acclimated. This is the first study on relative freezing tolerance of two different leaf ages indicating that this inherent tolerance is not dependent upon developmental stage. Maximum relative freezing tolerance was attained after 3 weeks of acclimation in both mature and young leaves, with young leaves showing a greater tolerance to freezing than the mature leaves. Although Uemura *et al* (1989) showed a similar response with greater

tolerance in younger leaves relative to mature rye leaves, no growth and development studies were done on these leaves. It is not known at what stage of development the leaves were in their study.

Following a change in temperature, the rates of growth of different parts of the plant may change relative to each other (Clarkson *et al*, 1988). Growth in turn is a consequence of cell division and cell expansion. In this study indeed it was found that growth parameters such as fresh weight, dry weight and leaf area differed in the two leaf ages during cold acclimation. Acclimation was associated with increase in dry matter and relatively low rates of cell division in mature leaves and increased dry matter accompanying high rates of cell division in young leaves. This is in contrast to the result of Cox and Levitt (1969) who showed that growth resulting from cell division was inversely related to freezing tolerance. Therefore, this is the first study which shows that active cell division plays an important role in cold acclimation.

Changes in the nucleic acid content were associated with acclimation in both leaf ages. No notable change in the DNA content was observed in the acclimated mature leaves, whereas in the acclimated young leaves a slight increase in DNA content was observed. This is the result of active cell division in young leaves. Numerous reports show increases in DNA content during cold acclimation(Teroka, 1973; Li and Weiser, 1969; Johnson-Flanagan and Singh, 1987, 1988). This suggests that previous studies have only examined young tissue or whole plant. As mature tissue is critical to the overall acclimation process, it is important to understand how they respond.

The effect of low temperature stress on some photosynthetic genes have been studied (Hahn and Walbot, 1989; Meza-Baso et al, 1986) but no studies have been done on the effect of low temperature acclimation on photosynthetic gene expression. Therefore this is the first study on the effect of cold acclimation on photosynthetic gene expression at the mRNA, protein and DNA level. The effect of low temperature acclimation was examined at the DNA, mRNA and protein level on four photosynthetic genes i.e. *lsu, ssu, cab* and *psbA* in both mature and young leaves. Young leaves showed higher expression of photosynthetic genes in comparison to mature leaves during cold acclimation. Examination of photosynthetic gene expression showed that

the two leaf ages differed. Low abundance of *ssu, cab, lsu* and *psbA* transcripts was associated with cold acclimation in mature leaves and high abundance in young leaves. In general, protein levels were high relative to the message level in mature leaves.

The observed differences in photosynthetic gene expression and protein accumulation between mature and young leaves during low temperature acclimation could suggest senescence in the mature leaves. This was not the case. Evidence from the four different studies, where the mature leaves showed increased freezing tolerance, increased both nuclear and chloroplastic rRNA expression, greater dry matter accumulation, increased protein content, lack of changes in chlorophyll a/b ratio and lack of LSU and SSU polypeptide turnover during the 10hr chase period all indicate a lack of senescence. At this time it is not clear why the photosynthetic transcript abundance was low. Further research is required on turnover of photosynthetic mRNAs to see whether posttranscriptional regulation led to low transcript abundance.

Antenna size of LHCII decreased in acclimated mature leaves, but did not in young leaves. This suggests that mature leaves may dissipate the excess light energy by reducing the number chlorophyll molecules per LHCII polypeptides. Decreases in antennae size of LHCs have been observed in potato during cold acclimation and are expected to be providing a protection against photo-oxidation (Steffan and Palta, 1987).

Growth and development at cold hardening temperature have shown that the chlorophyll content decreases on a dry weight basis in rye (Huner *et el* , 1984) and chlorophyll a/b ratio have also shown to be unaltered during growth and development at cold hardening temperatures (Krol and Huner, 1985). Similar observation were made in *Brassica napus* during three weeks of cold acclimation in this study. This indicates that the regulation of chlorophyll synthesis during cold hardening temperatures in rye and in *Brassica napus* may be similar.

A positive correlation between increased nuclear rRNA expression and cold acclimation have been associated in a number of plants (Steffan and Palta, 1986; Sarhan and Chevrier, 1985; Sarhan and D'Aoust, 1975; Paldi and Devay, 1977, 1983; Zverva and Turnova, 1985; Laroche *et al,* 1992). Results

from the present study supports this, as the young leaves had higher freezing tolerance and higher rRNA levels. Expression of chloroplast rRNAs have not been studied during cold acclimation and this study for the first time clearly showed that similar increases in chloroplast rRNA expression occurs during three weeks of low temperature acclimation. This indicates that the protein synthesizing machinery in chloroplast are also altered during cold acclimation. These results suggest that both cytoplasm and chloroplasts are actively involved in protein synthesis during cold acclimation.

There is a general requirement for protein synthesis for the induction of cold acclimation (Chen *et al*, 1983). This indicates changes in rRNAs, mRNAs and possibly changes in the local structure of the genes involved in protein synthesis. Changes in the local structure of a gene would alter the availability of the gene to RNA polymerases for transcription. One of such changes is methylation. There are a number of reports that show a correlation between transcription and methylation status in a number of photosynthetic genes during the conversion of chloroplast to chromoplast (Kobayashi *et al*, 1990), during tomato fruit development (Ngernprasirtsiri *et al*, 1988b), in amyloplasts (Ngernprasirtsiri *et al*, 1988b) and in mesophyll and bundle sheath cells of maize (Ngernprasirtsiri *et al*, 1989) but no studies have been carried out on the effect of low temperature acclimation on methylation status of photosynthetic genes.

The ribosomal genes and photosynthetic genes were examined for changes in methylation status during cold acclimation. Studies on two leaf ages revealed developmental differences in methylation where hypomethylation was generally associated with young leaves and hypermethylation in mature leaves. Alteration of methylation of specific sequences was suggested to be important during plant development by Watson *et al* (1987). Methylation changes were suspected as a possible mediator for contributing to differential expression of rRNAs in both leaves during cold acclimation. In general, both chloroplastic and nuclear rDNAs showed internal cytosine methylation at CCGG sequences in their ribosomal genes in both mature and young leaves. This internal cytosine methylation did not change with acclimation. This is the first report where hypomethylation at *Pst I* sites of chloroplastic rDNA could be correlated with rRNA expression during cold acclimation in both leaves. In addition, in the

non-acclimated plants, hypermethylation at *Pst I* site in mature leaves and hypomethylation at *Pst I* sites in young leaves were observed, indicating possible developmental control. Similarly, hypomethylation of rDNAs have been reported during development of *Xenopus laevis* (Bird *et al*, 1981) and hypomethylation of 16S rRNA gene has been correlated to its active transcription during tomato fruit development (Ngernprasirtsiri *et al*, 1888a). In contrast, nuclear rDNA did not reveal any methylation differences in either ages of leaf with acclimation.

Qualitative and quantitative methylation differences in the chloroplast encoded genes and nuclear encoded genes were observed for the first time in both acclimated and non-acclimated mature and young leaves. In general, the quantity of methylation decreased with acclimation in both leaves and was higher in mature leaves. Methylation difference were observed with acclimation for various genes. The relationship between gene methylation and transcript abundance was positive in *Msp I* hybridization patterns of *ssu*. Examination of *ssu* showed a very loose correlation between hypermethylation at *Pst I* sites and higher transcript abundance. A similar loose correlation was apparent between *cab* methylation at *Hind III* sites and *cab* levels. These results suggests that further examination of methylated sites at different sequences are required to understand the potential role of methylation for controlling gene expression during cold acclimation.

This study for the first time showed that there was a lack of co-ordination at the mRNA level but not at the protein level between LSU and SSU during low temperature acclimation. This suggests no limitation imposed on the capacity for carbon metabolism. It also suggests post-translational control.

CONCLUSIONS AND SPECULATIONS

Acclimation in mature leaves was associated with increased rRNA and increased protein synthesis. Despite this, both nuclear -encoded and chloroplast-encoded photosynthetic gene expression was suppressed. Methylation of the nuclear and chloroplast genes was correlated with this suppression. However, careful examination of the relationship on a gene for gene basis indicated that there was no clear relationship between hypomethylation and reduced transcription.

The low transcript levels may reflect a lack of protein turnover and a low growth rate. Low turnover rates have been associated with low temperature (Hahn and Walbot, 1989). Certainly, results from this thesis failed to show turnover. Despite the lack of co-ordination at the mRNA level, the polypeptides of LSU and SSU accumulated in proportion. This suggests that acclimated mature leaves have not lost their capacity for carbon metabolism. Measurements of leaf area confirmed a relatively slow growth rate. Perhaps, therefore, the drop in chlorophyll in the absence of changed chlorophyll a/b ratio is a significant clue. Such an observation suggests that chloroplasts are not differentiating during acclimation. The plastids that are present at the onset of acclimation, may be retained, as indicated by the relatively high levels of chloroplast proteins and the unchanged chlorophyll a/b ratio.

In contrast to the mature leaves, the young leaves were actively growing as indicated by thymidine incorporation and increases in leaf area during four weeks of cold acclimation. The young leaves, too, showed increases in rRNA with acclimation, but in this case, this was associated with increased transcription of the nuclear and chloroplastic encoded photosynthetic genes. In general, protein accumulation reflected the transcript levels. This suggested that the plastids are continuing to develop during the acclimation period. Even though nuclear encoded *ssu* genes showed higher expression than *lsu* but at the protein level LSU and SSU accumulated in proportion. This suggests no limitation on carbon metabolism in acclimated young leaves. Again, methylation status was correlated with acclimation, and in fact, hypomethylation was not only

correlated with acclimation in young leaves, it was correlated with the higher degree of acclimation seen between the two leaf ages. However, once again, careful examination showed that there was a lack of correlation between transcript levels and the degree of gene methylation. Only methylation at *Msp I* sites of *ssu* genes in mature and young leaves correlated with *ssu* transcript abundance during cold acclimation.

FUTURE DIRECTION

There are a number of questions raised as a result of this thesis: What are the functional implications of the lack of co-ordination of *lsu* and *ssu*? What other transcriptional controls are involved in mediating gene expression? How do post-transcriptional events regulate relative polypeptide accumulation? Future direction of this research leads into several studies: 1. Examination of the photosynthetic capacity and photosynthetic efficiency of mature and young leaves during cold acclimation. 2. Further research on feedback inhibition of metabolites on photosynthetic gene expression is required to establish the photosynthetic capacity of these two leaves during cold acclimation. 3. *In vitro* transcription studies on methylated genes should give better understanding of the potential role of methylation in cold acclimation. 4. Further examination of methylation should give a clear picture of the observed lack of correlation of methylation status to transcript abundance during low temperature acclimation. 5. mRNA turnover studies should reveal the differences in transcript abundance that were observed in the two leaves during cold acclimation 6. Polysome translations studies should give a better understanding of the relative transcript abundance to relative polypeptide accumulation in the two leaves.

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APPENDIX-1

Probes used for northern and southern analysis:

CLONE **REFERENCES**

pSS65 -pea *ssu* (cDNA containing 1-100 nucleotides encoding 33 amino acids of the transit peptide. The mature polypeptide is encoded by 101-468 nucleotides and there is 148 nucleotide 3' noncoding sequence preceding the poly (A) tail). Corruzzi *et al*, 1983

pMBB1.83 - mung bean *psbA* (genomic clone *Pst1* 800bp insert)

Palmer *et al*, 1982

pJZA4 -spinach *lsu* (genomic clone*Bam HI-Ava I* 1.9kb insert)

Erion *et al*, 1981

pAB56 -pea *cab* (The first methionine codon 3' from this site is 69 nucleotides away and is the initiating codon of the open reading frame. The "TATA" sequence occurs 31 nucleotides 5' from the cap site. *EcoRI-Hind III* 1000bp insert).

Cashmore, 1984

pMF2 -*Neurospora crassa*17S, 5.8S and 25S rRNA (7kb *Pst I* insert was used)

Free *et al*, 1978

pUC11-5*Brassica napus* cDNA containing 99% homology with with tobacco chloroplast 23S, 16S and intergenic spacer of 4.5 S rRNA (cloned at Eco RI site) Johnson-Flanagan(unpublished)

Polyclonal antibodies used in the Western blot analysis:

Antibodies	**Source**
RUBISCO of maize (raised in rabbit)	Dr. S. Hemmingson
LHCII of wheat (raised in rabbit)	Dr. M. Duysen; Dr. S. Gepstein
PSBA of barley (raised in rabbit)	Dr. J. Mullet

Primers used in PCR

pBR322-Pst I, (+) sequencing	d'(GCTAGAGTAAGTAGTT), 16-mer
pBR322-Pst I, (-) sequencing	d'(AACGACGAGCGTRGAC), 15-mer
pUC11-5- EcoRI (+) sequencing	d'(ACCATGATTACGAATTCG), 18-mer
pUC11-5- EcoRI (-) sequencing	d'(CCGAGCTCGAATTCACTG), 18-mer